Selective killing of oncogenically transformed cells by arsenic trioxide and trolox.

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ABSTRACT

A major disadvantage of cytotoxic chemotherapy is the difficulty in selectively targeting tumor cells. Therefore, understanding the biological differences between cancer and normal cells provides a foundation for designing strategies to improve cancer therapy. Although arsenic trioxide (ATO, As_2O_3) is an effective therapy in acute promyelocytic leukemia (APL), its use in solid tumors is limited by the toxicity observed at concentrations required to induce apoptosis in non-APL cells.

It is well characterized that oncogenically transformed cell lines generate a greater amount of reactive oxygen species (ROS) at basal levels.[1,2] Furthermore, it is thought that the increase of ROS formation renders the transformed cells more sensitive to additional oxidative stress.[3] Previous data from our lab show that cell death caused by ATO is a result of the accumulation of free radicals, which in turn, results in oxidative stress. Moreover, we previously found that trolox, a hydrophilic vitamin E analogue lacking the phytyl tail, enhances ATO-induced ROS and apoptosis in APL, myeloma, and breast cancer cells, while simultaneously protecting normal cells from arsenic-induced toxicity.[4,5] The objective of the current study is to understand the mechanism(s) by which trolox switches from a cytoprotective function to a cytotoxic function upon oncogenic transformation of cells.[6]

In order to study the effects of ATO in combination with trolox, two isogenic cell line models were used. An isogenic cell model includes two cell lines with identical genomic background with the exception of one of the cell lines being oncogenically transformed. In this thesis, we compared the wild type mouse embryonic fibroblast (MEF) cell line to MEF cells transformed with either E6/E7 or E1A/Ras oncogenes. The effects of arsenic alone and in

combination with trolox were investigated in both isogenic models. Trolox potentiates ATO-induced toxicity in malignant cell lines, but has the opposite effect in their normal counterparts. Our data suggests that this difference in effect may be due to disruption of normal mitochondrial function. We speculate that the mitochondrial dysfunction is related to the efficiency of the mitochondrial complexes that make-up the electron transport chain (ETC). Our findings may demonstrate how combination therapies such as ATO and trolox can be used to achieve such activity *in vitro*, with the potential to extend to *in vivo* studies.

RÉSUMÉ

Un des inconvénients majeurs de la chimiothérapie est de sélectivement cibler les cellules cancéreuses. D'abord, la compréhension des différences biologiques entre les cellules cancéreuses et saines permet de créer des stratégies qui visent à améliorer le traitement pour le cancer. Le trioxyde d'arsenic (ATO, As2O3) est un traitement efficace pour la leucémie promyélocytic aigüe (APL), cependant, l'utilisation de l'ATO contre d'autres cancers est limitée puisque les doses effectives pour induire l'apoptose deviennent toxiques pour les cellules saines.

Les cellules malignes ont démontré de posséder une grande capacité d'oxydation.[1,2] On émettent l'hypothèse que l'augmentation de la formation d'espèces radicalaires de l'oxygène rend les cellules transformées plus sensibles aux stress oxydatif supplémentaire.[3] Certaines données de notre laboratoire montrent que l'effet cytotoxique provoqué par l'ATO est le résultat de l'accumulation des radicaux libres intracellulaires, qui ajoute un stress oxydatif dans les cellules cancéreuses. Nous avons déjà constaté que trolox (acide 6-hydroxy-2,5,7,8-tétraméthylchroman-2-carboxilique) améliore l'effet cytotoxique de l'ATO dans les cellules malignes étudiées *in vitro* comme le myélome, et les cellules de cancer du sein. De plus, cette combinaison protège les cellules saines des effets toxiques de l'ATO.[4,5] L'objectif de la recherche présenté dans cette thèse est de comprendre les effets différentiels de l'ATO en combinaison avec trolox selon le type de cellule.[6]

Afin d'étudier ces effets, deux modèles de lignées cellulaires isogéniques ont été utilisés. Un modèle de lignées cellulaires isogéniques comprend de deux lignées cellulaires avec un

génome identique avec l'exception qu'une des lignées cellulaires ont une transformation oncogénique. Dans cette thèse, nous avons comparé les fibroblastes embryonnaires murins avec les fibroblastes embryonnaires murins transformées oncogéniquement avec soit oncogènes E6/E7 ou E1A/Ras. Comme auparavant dans notre laboratoire, les expériences avec les fibroblastes embryonnaires murins montrent que le trolox potentialise le stress oxydatif dans les cellules malignes et, en même temps, protège les cellules saines de la toxicité engagé par l'ATO. Nos données suggèrent que cette différence d'effet peut être à cause des fonctions des mitochondries. Les résultats de recherche présentés dans cette thèse, suggèrent que le trolox en combinaison avec l'ATO a des effets significatifs *in vitro* et pourrait avoir le potentiel d'être utilisée *in vivo*.

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CONTRIBUTIONS OF WORK

The candidate performed the majority of the research described in this thesis, with the support of Dr. Wilson H. Miller. The contribution of other authors to this work is as follows:

In Chapter 3:

M.Sc. Stanley Kwan provided assistance with the Ras western blot in Figure 1C.

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ABBREVIATIONS

ABBC1:	ATP Binding Cassette Protein
AMPK:	AMP-Activated Protein Kinase
Apaf-1:	Apoptotic Protease Activating Factor 1
APL:	Acute Promyelocytic Leukemia
ATBC:	Alpha-Tocopherol, Beta Carotene
ATO:	Arsenic Trioxide
ATP:	Adenine Triphosphate
ATRA:	All-Trans Retinoic Acid
Bak:	BCL-2 Homologous Antagonist/Killer
Bax:	BCL-2-Associated X
BCL-2:	B-Cell Lymphoma 2
CAC:	Citric Acid Cycle
dATP:	Deoxyadenosine Triphosphate
DISC:	Death-induced Signaling Complex
DRP1:	Dynamin-Related protein
ECAR:	Extracellular Acidification Rate
ETC:	Electron Transport Chain
FADD:	Fas-associated Death Domain protein
FADH2:	Flavin Adenine Dinucleotide
FCCP:	Trifluorocarbonylcyanide Phenylhydrazone
GLUT:	Glucose Transporter

HO-1: Heme Oxygenase-1

HPV: Human papillomavirus

JNK: C-Jun N-terminal Kinase

MEF: Mouse Embryonic Fibroblast

MMP: Mitochondrial Membrane Potential

NADH: Nicotinamide Adenine Dinucleotide

OCR: Oxygen Consumption Rate

PKA: Protein Kinase A

PBMC: Peripheral Blood Mononuclear Cell

PI: Propidium Iodide

RIP: Receptor-interacting Protein

ROS: Reactive Oxygen Species

SELECT: Selenium and Vitamin E Cancer Prevention Trial

SOD: Superoxide Dismutase

TCA: Tricarboxylic Acid

TEM: Transmission Electron Microscope

TNF: Tumor Necrosis Factor

TRADD: TNF Receptor-Associated Death Domain protein

ULK1: Unc-51-like kinase 1

WHO: World Health Organization

CHAPTER 1: LITERATURE REVIEW

1.1 Cancer

Cancer comprises a broad spectrum of diseases consisting of abnormal cells that divide and grow in a deregulated fashion. Classical hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, invasion and metastasis, enabling replicative immortality and inducing angiogenesis. Emerging characteristics of cancer cells consist of deregulating cellular energetics, avoiding immune destruction, genome instability and tumor-promoting inflammation. The main categories of cancer include carcinoma, sarcoma, leukemia, lymphoma and myeloma.[7]

Cancer is the leading cause of death worldwide. In Canada, it is estimated that 2 out of 5 people will develop this disease over their lifetime and that 1 out of 4 Canadians will die from cancer.[8] Unfortunately, the incidence of cancer amongst individuals continues to increase progressively: prostate cancer being the most commonly diagnosed cancer in men and breast cancer is the most common type of cancer diagnosed for women. Lung cancer has the highest mortality rate in both women and men.

The progressive transformation that occurs within a normal cell to become a malignant cancer cell is dependent on the genetic background of the cell as well as environmental factors. Exposure to sun, cigarette smoke, stress, infections, environmental pollutants, unhealthy diets and hormones are collectively known as ‘risk factors’, which contribute to the increasing chance

of a malignant transformation. Furthermore, age is listed as the most important risk factor due to an accumulation of mutations over time and a decreasing efficiency of repair mechanisms.[9]

Established treatment protocols for cancer include surgery, radiation, chemotherapy and hormone therapy. Considerations such as the type of cancer, the stage of the disease, and the physical well being of the patient are required when planning for the optimal treatment. [10] A major disadvantage of using cytotoxic chemotherapy is the difficulty in selectively targeting tumor cells. Therefore, understanding the biological differences between cancer and normal cells provides a foundation for designing strategies to improve cancer therapy.

1.2 Arsenic Trioxide as a Treatment

1.2.1 Physical and Chemical Properties of Arsenic and Arsenical compounds

Arsenic is classified as the 33rd element and, therefore, it is a group V metalloid. Consequently, arsenic possesses both metallic and non-metallic properties. Arsenic is the 53rd most abundant element in the earth's crust: Soil contains 1.5-2 ppb of arsenic[11] and seawater has only 1.6 ppb arsenic.[12] Arsenic can exists in both inorganic and organic forms; each having particular chemical, physical and cytotoxic properties. The three most common inorganic forms are arsenic trisulfide (As_2S_3), arsenic disulphide (As_2S_2) and arsenic trioxide (As_2O_3, ATO). These common arsenical compounds are also referred as yellow, red and white arsenic respectively.[13,14]

Arsenic Trisulfide (As_2S_3)[15] Arsenic Disulphide (As_2S_2)[16] Arsenic trioxide (As_2O_3)[17]

Figure 1.1 Types of inorganic arsenic include As_2S_3, As_2S_2 and As_2O_3.

1.2.2 History of Arsenic as a Treatment

Although arsenic is widely renown as a poison, it has also been recognized as a treatment for the past 2400 years. As early as 200 B.C., during the Han (漢) dynasty, Traditional Chinese Medicine mentions the use of two arsenicals: arsenic sulphide (A_2S_4) and arsenic trioxide (ATO). The rational of the use of these hazardous compounds was because it was thought that poisons in small doses would be able to realign the internal equilibrium within a person. Therefore, at the time, arsenic was used to treat jaundice, parasitic infections and malignant growths. [18]

In the 18th century, Thomas Fowler, an English scientist, developed a potassium bicarbonate-based solution of arsenic trioxide known as "Fowler's Solution" to treat many diseases such as hypertension, heartburn and chronic rheumatism. It was only in 1878, in a hospital in Boston, that Fowler's solution was found to have a reduction effect on the number of white blood cells in a leukemic patient. It would remain a popular chemotherapeutic agent throughout the 19th and

the 20th century. In 1930, the efficacy of ATO was reported in chronic myelogenous leukemic patients. Other diseases, including syphilis[18] and psoriasis[19] also tried ATO as a remedy. By the end of the 20th Century, the Chinese reported the beneficial uses of ATO in acute promyelocytic leukemia (APL). [18,20] Consequently, confirmation of ATO's therapeutical uses in APL in other reports led to the approval of Trisenox (ATO containing substance) as a treatment by the FDA.[11,21] ATO is delivered to the patient in low doses (0.5–2.0μM) to induce differentiation and apoptosis of leukemic cells. [22]

Importantly, in 2013, it was demonstrated that ATO, when paired with all-trans retinoic acid (ATRA) (a derivative of Vitamin A), could be used as a first line treatment for acute promyelocytic leukemia (APL), eliminating the use of other chemotherapies.[23,24] Although arsenic trioxide is an effective therapy in APL, its use in solid tumors is limited by the toxicity observed at higher concentrations (8μM) required to induce apoptosis in non-APL cells. [25,26]

1.2.3 Toxicity of Arsenic

Arsenic is naturally found in the water, soil and rocks. Furthermore, it can be released into the atmosphere, usually as a trioxide compound, by means of high-temperature processes such as volcanic activity. Human exposure to arsenic has risen because anthropogenic procedures like mining, metal smelting, burning of fossil fuel and agricultural pesticide production induce the release of arsenic in both the water and atmosphere as well. Soluble inorganic arsenic exposure is highly toxic and arsenic is naturally found at high levels in drinking water in numerous countries and creating a significant threat to the public health in China, India, Mexico, Argentina and in

particular, Bangladesh. In 2001, it was estimated that the death of about 9100 Bangladesh civilians was the result of drinking arsenic-contaminated water.[27] Other human exposures to arsenic include smoking and ingestion of food prepared in high-arsenic water, such as rice.[28] After evidence of arsenic-related toxicities around the world, in 1993 the World Health Organization (WHO) determined that 10 μg/L is the acceptable concentration of arsenic in drinking water, [29] although, many countries have yet to adopt this safety guideline.

The absorption and metabolism of arsenic depend on the form in which is ingested. Soluble inorganic arsenic poses as a great health risk because it is quickly absorbed from the into cells via aquaporins and glucose transporters (GLUT) 3,7 and 9.[30] Once absorbed, the compound undergoes a series of biochemical reactions, which is predominantly executed in liver cells. Arsenic undergoes reactions such as reduction by cytochromes in the cytoplasm and methylation by arsenic (III) methyltransferase to form pentavalent organic intermediates. Furthermore the arsenical is modified into a water-soluble isoform to facilitate excretion via ATP binding cassette protein (ABBC1) and multi-resistant drug protein (MRP) transporters.[31,32] It has been suggested that toxicity of arsenic increases with increasing methylation of arsenic.[33] Excretion of arsenic is predominantly completed via urination or defecation.[34]

Health risks of arsenicals have significant importance to the public since its toxic characteristic has been exploited as a treacherous tool in homicides in the past.[18,35] However, arsenic related-toxicity depends on various factors, which include the duration of the exposure and the chemical structure of the arsenical. Unhealthy effects arising from arsenicals can be the result of either acute or chronic exposure. Effects of acute arsenic poisoning include vomiting, gastrointestinal

discomfort and diarrhea. This may lead to electrolyte imbalance, hypertension and hypoxia. Subsequently, multi-organ failure may arise and can cause lethal consequences.[29,33] Nowadays, chronic exposure to arsenic, such as the ingestion of contaminated drinking water, is of greatest concern for arsenic toxicity. Chronic toxicity is the result of long-term exposure to various doses of arsenic. Symptoms such as changes in skin pigmentation, renal failure, hypertension and gangrene are due to chronic exposure of high inorganic arsenic levels.[29] Data also suggests that long-term toxicity may cause cancers of the skin, bladder and lungs.[36,37,38] Furthermore, arsenic can pass through the placenta and may induce spontaneous abortion, stillbirth and health complications for newborns.[29]

1.3 Apoptosis

Apoptosis is the programmed self-destruction of the cell that occurs in various physiological and pathological contexts. Apoptosis is necessary to maintain organ and tissue homeostasis: damaged or superfluous cells are eliminated to control cell number. Programmed cell death is characterized by a collection of events: it involves rapid shrinkage of the cell, double-strand cleavage of nuclear DNA and membrane blebbing. In contrast to necrosis, these morphological changes eventually induce the formation of apoptotic bodies containing organelles, which are engulfed and digested by macrophages. As a result of phagocytosis by the macrophage, there is a limited amount of associated inflammation to neighbor cells. This process may occur intrinsically (mitochondrial mediated) or extrinsically (death receptor mediated),[39] however, these pathways are not mutually exclusive because both involve the use of intracellular cysteine

proteases called caspases. Dysfunction of apoptosis of cells leads to multiple diseases including cancer.[40,41,42]

1.3.1 Apoptotic Pathways

1.3.1.1 Intrinsic Apoptosis

Intrinsic apoptosis, also known as mitochondria-dependent apoptotic pathway, is defined as the permeabilization of the mitochondria and the subsequent release of cytochrome c to activate caspases, which in turn, induce cell death.[43] Mitochondrial involvement in apoptosis is key in programmed cell death via the intrinsic pathway. B-cell lymphoma 2 (BCL-2), a pro-survival related protein, is located primarily on the outer membranes of mitochondria. When BCL-2 is over-expressed, cellular apoptosis from various stimuli is inhibited. It suggests that the presence of mitochondria is central to apoptosis.[44,45] Subsequently, studies have shown that induction of caspase activation by addition of deoxyadenosine triphosphate (dATP) depended on the presence of cytochrome c released from mitochondria during the initial phases of apoptosis.[46] It has been demonstrated that cytochrome c release from mitochondria can be inhibited by BCL-2. [47,48] Once cytochrome c is released, it binds to Apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 to induce the cleavage and activation of caspase 9.[49] Consequently, activating caspase-9 results in the cleavage of caspase-3, which in turn activates the programmed cell death.[41,42]

1.3.1.2 Extrinsic Pathway

The extrinsic pathway initiates apoptosis with the use transmembrane receptor-mediated interactions. In specific, these "death receptors" are members of the tumor necrosis factor (TNF) receptor gene superfamily.[50] TNF receptors possess cysteine-rich extracellular domains and a cytosolic sequence of 80 amino acids. The cytosolic domain of the receptor is crucial for transmitting the signal to trigger intracellular pathways leading to apoptosis, and so it also known as the "death domain".[51] Well-known ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. Ligands can bind to their respective receptor in monomeric fashion or in clusters such as dimers or trimers.[52] Once bound to the receptor, ligand-receptor interactions trigger the recruitment of proteins to form a death-inducing signaling complex (DISC). In the example of Fas/FasR, Fas-associated death domain protein (FADD) is recruited along with other proteins such as TNF receptor-associated death domain protein (TRADD) and receptor-interaction protein (RIP).[53] In turn, this complex mobilizes procaspase-8 to the death receptor and this results in the autocatalytic activation of procaspase-8.[54] Activation of the caspase-8 leads to the cleavage of caspase-3. Similar to intrinsic apoptotic pathway, caspase-3 activation induces many reactions to destroy the cell.[42]

1.3.2 The Role of ATO in Apoptosis

It has been reported that ATO induces apoptosis in APL and non-APL hematological malignancy and solid tumor cells, however, higher concentrations are required to cause cell death in non-APL contexts. [55,56] Several mechanisms are implicated during ATO-related apoptosis. Studies

have shown that ATO induces the accumulation of free radicals and subsequent generation of reactive oxygen species (ROS)[57]. Previously in our lab, we have shown that administration of ATO is cytotoxic and induces upregulation of heme oxygenase-1 (HO-1), an oxidative stress marker, in normal and several cancer cell lines.[58,59] Other intracellular events, such as down-regulation of BCL-2 expression,[60] cell cycle arrest [61] and permeabilize the mitochondrial membrane[62], also contribute to cell death. Furthermore, our lab has demonstrated that ATO-related apoptosis requires the activation c-jun N-terminal kinase (JNK) in APL hematological cells.[57]

1.4 Antioxidants

As a byproduct of metabolic pathways, ROS are formed in live cells. Many reports discuss the role of ROS and their effects in biological systems. [3,63,64] Subsequently, ROS produce free radicals within the cell and in turn, these radicals can start chain reactions, resulting in cellular damage.[65] The cell's natural defense mechanism to ROS is through the use of antioxidants.[66] Antioxidants bring these chain reactions to an end by removing free radical intermediates, and inhibiting further oxidation reactions. Termination of free radical chain reactions is achieved when the antioxidant itself becomes oxidized, therefore, antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols. Inhibition of antioxidants leads to chronic inflammation.[67] Natural sources of antioxidant include fruits, vegetables. Previous studies have investigated the beneficial effect of antioxidants and their preventive effect of diseases such as cancer, coronary heart disease and even altitude sickness.[68] However, conflicting results seen in recent clinical trials suggest that there is in fact no benefit in incorporating antioxidants as a

preventative treatment and even propose that excess supplementation with certain antioxidants may be harmful.[69,70] Antioxidants also have many industrial uses, such as dietary supplements, preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline.

1.4.1 Physical and Chemical Properties of an Antioxidant

Antioxidants are classified based on their structure, solubility and kinetics. Categories of antioxidant include: carotenoids, enzymes, glutathione, hormones, lipid associated chemicals, minerals, phenolics, steroids and vitamins.[67] Furthermore, within each group, antioxidants can be classified into two broad divisions depending on their solubility in water. Hydrophilic antioxidants react with oxidants in the cytoplasm, while, lipid-soluble antioxidants protect membranes from lipid peroxidation.[67] Kinetically, antioxidants can be categorized into two sub-divisions: enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), catalases and glutathione peroxidase. The antioxidant properties of these enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction, therefore, inhibiting the oxidation of ferrous iron (II) by hydrogen peroxide and, thus, the formation of hydroxyl radicals and hydroxyl anions. In turn, organic compounds within the cell are protected from the free radicals generated in this process. Enzymatic antioxidants remove free radicals by converting them into hydrogen peroxide, and subsequently into water with the help of metal cofactors (copper, zinc, manganese and iron). Non-enzymatic antioxidants include vitamin C, vitamin E, carotenoids, thiol antioxidants, flavonoids and melatonin. These compounds do not remove free radicals but, instead, terminate their chain reactions.[71] These protective compounds can either be synthesized endogenously or acquired by food intake. Although certain

antioxidants are mostly found within the cell and other protective compounds, are distributed more evenly through out the bodily tissues, all antioxidants help to control levels of oxidative stress and act as a coordinated system.[72]

1.4.2 Vitamin E

Vitamin E is collective name for tocopherols and tocotrienols, which are fat-soluble non-enzymatic antioxidants. Vitamin E's main function is protection against lipid peroxidation. The protective mechanism is completed by the interruption of the free radical chain reactions. The hydrogen from the aromatic ring group is donated to the free radical, resulting in a relatively stable free radical form of the vitamin. [71,73]

1.4.2.1 Clinical Use of Vitamin E

In 1922, Herbert McLean Evans and Katharine Scott Bishop discovered Vitamin E. It was first isolated in a pure form by Gladys Anderson Emerson in 1935 at the University of California, Berkeley. In 1938, the first clinical use for vitamin E as a therapeutic agent was conducted by Widenbauer.[74] Briefly, vitamin E was given to 17 premature newborn infants suffering from growth failure. Eleven of the original 17 patients recovered and were able to resume normal growth rates. The characterization of the therapeutical effects of vitamin E was further investigated in 1945 when Drs. Evan V. Shute and Wilfred E. Shute published the first findings arguing that high doses of vitamin E can prevent the development of atherosclerosis.[74,75,76] At the turn of the 21st century, vitamin E was beginning to be defined for its therapeutical use in cancer

[77] and in particular, prostate cancer. [78,79,80] However, data from these trials, amongst others[81,82], have conflicting results in terms of the beneficial effect of this antioxidant. The Alpha-Tocopherol, Beta Carotene (ATBC) trial indicates that vitamin E supplementation lowers the incidence of prostate cancer. However, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) demonstrate that Vitamin E is not a beneficial treatment option for prostate cancer.

1.4.2.2 Trolox

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a hydrophilic vitamin E analog lacking the phytyl tail, causing the compound to have an increased antioxidant capacity due to an elevated diffusion rate into the cell. Trolox is well characterized as an antioxidant and having a protective role in cells against oxidative stress.[83,84] However, recent studies have shown that trolox also has a potentiating role in cytotoxicity, in particular when treated in combination with ATO.[4,5]

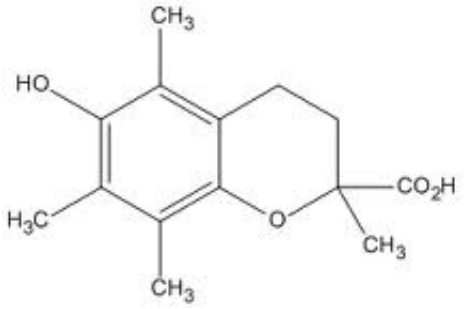


Figure 1.3 Chemical structure of trolox is similar to that of vitamin E. [85]

1.5 Mitochondria

Mitochondria are membrane-bound organelles found in eukaryotic cells. Mitochondria are comprised of a double bilayer of phospholipids and range from 0.5 to 1.0 micrometer in diameter. The mitochondria, found within each cell, alternate in configuration to form a dynamic network in order to carry out their function.[86] The main function of the mitochondria is to provide the cell with energy in the form of ATP.[87] The number and the size of the mitochondria influence the capacity of production of cellular energy within each cell.[88,89] Besides supplying ATP, mitochondria are involved in other functions such as cell signaling, cell cycle and cell death.[90] The condition of the mitochondria are central in several human diseases [91,92], including cancer [93], Alzheimer's [94], down-syndrome [95] and cardiac dysfunction [96,97].

1.5.1 Structure of the Mitochondria

The mitochondria consist of several components, which execute specialized functions. These include the outer mitochondrial membrane, the inter membrane space, the inner mitochondrial membrane, the cristae and matrix. The outer mitochondrial membrane encloses the organelle and disruption of this structure leads to cell death.[98] The inner mitochondrial membrane is folded within the organelle, which makes up the cristae. The space within this structure retains the matrix; proteins of the electron transport chain (ETC) and redox reactions of oxidative phosphorylation, and proteins necessary for mitochondrial fission and fusion. It also contains the structures essential for protein import and export of metabolites.[99,100] The inter membrane space retains the protons from which the potential generated is used to produce ATP.[101]

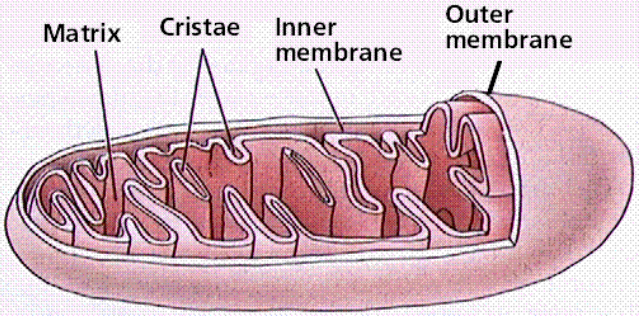


Figure 1.2 Structure of the mitochondria within eukaryotic cells.[102]

1.5.2 Role of the Mitochondria in the Cell

The structure of the mitochondria is very much related to its function. The folded inner mitochondrial membrane that make up the cristae, provide a large surface area to retain proteins needed for energy production. There are two main pathways to generate energy in the form of ATP within the cell: glycolysis and the ETC. Once glucose is transported into the cell, ATP is produced within the cytoplasm via glycolysis, also known as anaerobic respiration. However, substrates from the glycolytic pathway can propagate into the mitochondria and feed into the ETC to generate a greater number of ATP compared to glycolysis alone. This process is known as the aerobic respiration, since it requires the presence of oxygen as the final electron acceptor.[87,90] In addition to providing cellular energy, mitochondria are involved in other cellular functions, such as cell signaling and cell cycling.[103,104] The outer mitochondrial membrane is made up of phosphorylated lipids and acts as a binding platform for several proteins. A study shows that Rab32, a member of the Rab subfamily of Ras small molecular weight G-proteins, acts as an anchoring protein on the outer membrane of the mitochondria. It recruits protein kinase A (PKA) and proteins that are involved in mitochondrial dynamics, which regulate

membrane fusion/fission, exocytosis and cytoskeletal trafficking.[90,105,106] Other proteins, such as dynamin-related protein 1 (DRP1) that is needed for mitochondrial fission during apoptosis or cell cycling, are also tethered to the organelle due to the lipid content of the mitochondria.[90,107]

1.5.3 Mitochondrial Metabolism

As glucose enters the cell via glucose transporters (GLUTs), it is immediately phosphorylated by hexokinases to be kept inside the cell. The process to convert one molecule of phosphorylated glucose into two molecules of pyruvate, 2 nicotinamide adenine dinucleotides (NADH) and 2 ATPs is known as glycolysis.[108] Pyruvate is either fermented into lactate or actively transported into the matrix of the mitochondria, along with NADH. Pyruvate is then decarboxylated to form acetyl-CoA, which induces by-products: carbon dioxide and NADH. [109] The acetyl-CoA is the primary substrate to enter the citric acid cycle (CAC), also known as the tricarboxylic acid (TCA) cycle or Krebs cycle. With the exception of succinate dehydrogenase, which is bound to the cristae, the enzymes of the CAC are localized within the mitochondrial matrix. [110]The citric acid cycle consists of oxidizing the acetyl-CoA by combining it with oxaloacetate to form citrate, which is then subsequently chemically transformed by the CAC enzymes to produce 2 molecules of carbon dioxide, ATP, and several co-factors: three molecules of NADH and one molecule of flavin adenine dinucleotide ($FADH_2$). Due to the change of configuration of citrate and the loss of 2 carbons, the presence of oxaloacetate re-emerges at the end of one turn of the CAC. The co-factors, which were formed either in the cytoplasm or the CAC, will enter the ETC by reducing the complexes I-IV. In turn, the reduction of the complexes will result in translocating a hydrogen ion across the inner mitochondrial membrane and into the intermitochondrial space,

which creates a strong electrochemical gradient, known as the mitochondrial membrane potential (MMP). [111] The protons return into the matrix via the ATP synthase complex. This process is known as chemiosis, which consists of utilizing the potential energy generated by the MMP to synthesize ATP from ADP and inorganic phosphate (Pi).[112] This process is mostly efficient, however, protons that leak into the mitochondrial matrix, without passing through the ATP-synthase complex, will result in the formation mitochondrial superoxide.[113,114]

1.5.4 Role of Mitochondria during Apoptosis

Mitochondria are involved in the early stages of apoptosis. The subsequent activation of caspases in the mitochondrial-dependent apoptotic pathway requires the release of cytochrome c, and other proteins found in between the outer and inner membranes, into the cytoplasm. This is possible via the permeabilization of the mitochondrial outer membrane (MOMP).[98] Release of cytochrome c is regulated by a number of factors. Studies suggest that that mitochondrial fragmentation and the deterioration of cristae are crucial steps for cytochrome c release and cell death.[115,116] It has be shown that regulation of MOMP is also partially controlled by the presence of BCL-2. BCL-2 inhibits cytochrome c release by physically obstructing the formation of BCL-2-Associated X (Bax)/ BCL-2 Homologous Antagonist/Killer (Bak) lipid pore, which permeate the outer membrane to allow cytochrome c to be released. Once MOMP has occurred, cytochrome c recruits APAF-1 to trigger the formation of apoptosomes, and this leads to subsequent activation of the initiator and executioner caspases, caspase-9 and -3, respectively, which drives apoptosis.[98]

1.6 Isogenic Cell Line Models

Previously in our lab, the effects of arsenic trioxide related-therapies have been investigated in an APL model cell line, known as NB4.[4,5]Peripheral blood mononuclear cells (PBMC) were defined as normal cells and used as a control to then be compared to effects shown in NB4 cells. However, the cell types do not posses the same genetic background. In order to study the mechanistic differences between wild-type and cancer cells, an isogenic model should be used.[117] Isogenic models include a normal/wild-type cell line and an oncogenically transformed cell line. Both cell lines have the same genetic background except for the oncogenes, which induce the oncogenic transformation. Results that are obtained from this model are by definition 'matched' since the only different element is the additional transformation of one of the cell lines.

1.6.1 Oncogenic Transformation

Oncogenic transformation is a process by which normal cells are activated to behave as cancer cells. Oncogenes are transduced in normal cells and this procedure subsequently induces a phenotype change that occurs at the genetic and epigenetic level. Ultimately, it reprograms a cell to undergo uncontrolled cell division and adopt other cancer hallmarks.[118,119] More than one mutation is necessary for oncogenic transformation.[120]

1.6.2 Oncogenic Proteins

The two isogenic models used in this study uses mouse embryonic fibroblasts (MEF) either

transduced with E6 and E7 or E1A and Ras oncogenes. E6 protein encoded by the oncogenic human papillomavirus (HPV) types 16 and 18 is one of the viral proteins expressed in HPV-associated cancers. E6 cooperates with E7 to immortalize cell lines.[121] E6 oncoprotein induces the ubiquitination and subsequently the degradation of tumor suppressor protein p53. E7 oncoprotein induces the phosphorylation of Rb, therefore releasing the inhibition of cell cycle progression.[122] For the second isogenic cell model, oncogenically transformed MEFs were transduced with E1A and Ras. E1A induces the disassembly of the E2F1 transcription factors from RB1 by direct competition for the same binding site on RB1, with subsequent transcriptional activation of E2F1-regulated S-phase genes. Inactivation of the ability of RB1 to arrest the cell cycle is critical for cellular transformation, uncontrolled cellular growth and proliferation. Ras oncoprotein is part of the small GTPase family. It has 3 isoforms: H-Ras, N-Ras and K-Ras.[123] Once activated, receptor stimulate signal transduction events in the cytoplasm, a process by which proteins and second messengers relay signals from outside the cell to the cell nucleus and instructs the cell to grow or divide.[124] In addition to E1A, constitutively active H-Ras, with glycine to valine mutation at residue 12 renders the GTPase domain of Ras insensitive to inactivation, was also transduced into MEFs. In the second isogenic model, normal cells were transduced with the empty vector, pLPC.

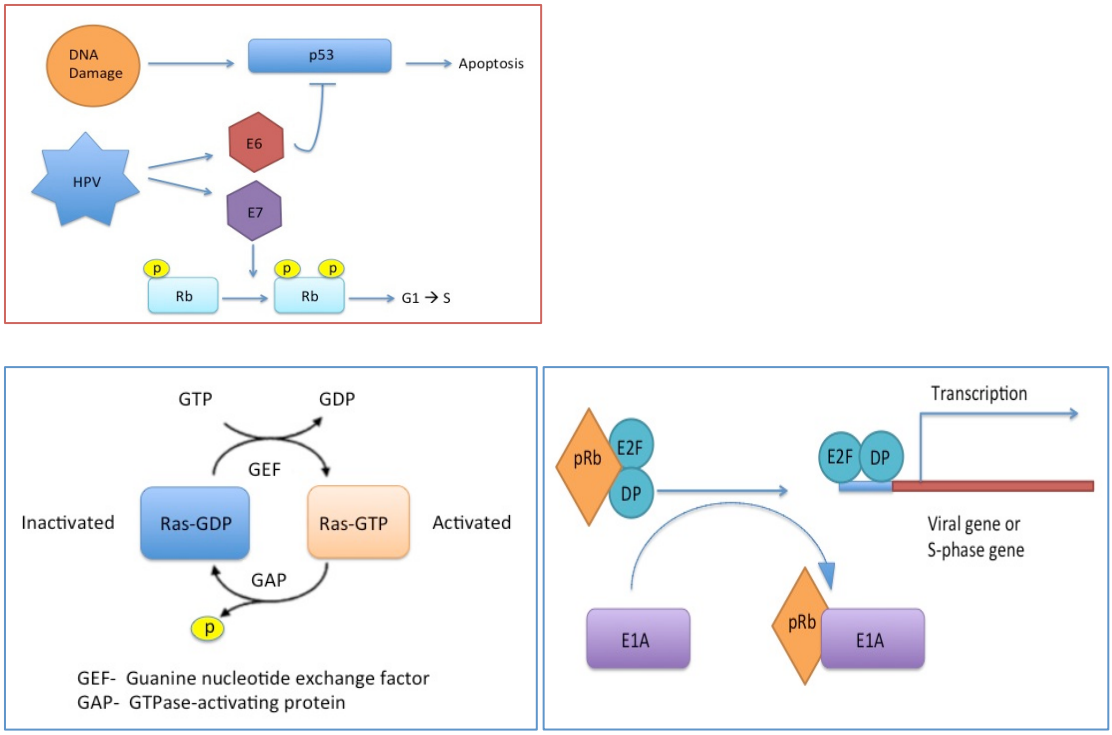


Figure 1.3 Oncogenic transformations in both isogenic cell line models.

1.7 Rationale and Objectives

Arsenic trioxide (ATO) is delivered to the patient in low doses (0.5–2.0μM) to induce differentiation and apoptosis of leukemic cells.[22] In 2013, it was demonstrated that ATO, when paired with all-trans retinoic acid (ATRA), could be used as a first line treatment for acute promyelocytic leukemia (APL), therefore, removing the use of other chemotherapies.[23,24] Despite the fact that ATO is an effective therapy in APL, its use in solid tumors is constrained by the toxicity observed at higher concentrations (8μM), which is required to induce apoptosis in non-APL cells. [25,26] Strategies are need to be developed in order to administer ATO to non-APL patients. In this thesis, we focus on using ATO in combination with another chemical agent and how it may prove to be effective for non-APL patients. We previously found that trolox, a

vitamin E derivative, enhances ATO-induced apoptosis in APL cells and P388 lymphoma mouse model, while simultaneously protecting normal cells. [4,5]

Wild-type MEFs and oncogenically transformed MEFs provide an isogenic model to compare the differences in effect of the treatment of ATO in combination with trolox. In this thesis, we aimed to further elucidate the cytotoxic effect of ATO in combination with trolox. Our specific objectives were as follows:

(1) To investigate the cellular effects of ATO administered in combination with trolox in both isogenic models.

(2) To examine the change in mitochondrial structure and efficacy in function when treated with ATO in combination with trolox.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines

Mouse Embryonic Fibroblasts (MEFs) and MEFs stably transfected with either oncogenes E6/E7 or E1A/Ras were maintained in DMEM at 37°C and 5% CO2. MEFs and MEFs transfected with the oncogenes E6 and E7 (MEFs E6/7) were kindly obtained from Dr. Al Moustafa. MEF pLPC and MEF E1A/Ras were created via viral transduction (Stanley Kwan, unpublished). Positively transduced cells were selected in media supplemented with 2μg/ml puromycin. All media were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada) and supplemented with 10% fetal bovine calf serum (FBS).

2.2 Proliferation Assays

MEF, MEF E6/7, MEF pLPC and MEF E1A/Ras were seeded at $5x10^4$ cells/ml in 6-well plates. Cells were counted on Day 1, 3 and 6. This was completed in triplicate and viable cell exclusion was completed by staining the samples with trypan blue. All cells were maintained at a density lower than $1x10^6$ cells/ml through dilution as required.

For the treatment proliferation assay, all cell lines were seeded at $7x10^5$ cells/ml in 6-well plates. Cells were treated with fresh media containing 5μM ATO, alone or in combination with 100μM trolox for six days. The number of viable (trypan blue excluding) cells was counted on Day 1, 3

and 6 in triplicate. All cells were maintained at a density lower than $1x10^6$ cells/ml through dilution as required, and media +/- treatment was replaced every third day.

2.3 Propidium Iodide

Cells were seeded at 1.5 x 10^4 cells/ml in 6-well plates at a density of 1.5 x 10^4 cells/well. The next day, fresh media was added and cells were treated with 5μM ATO alone or in combination with 100μM trolox for 48 hours. Cells were washed in buffer (PBS/ 5% FBS/ 0.01M NaN_3) at 4^oC, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured on a Becton-Dickinson FACS Calibur. SubG0 cell population results were obtained by staining the cells with propidium iodide followed by analysis using CellQuest software.

2.4 MitoSOX Staining

Cells were seeded in 6-well plates at a density of 1.5 x 10^4 cells/well. The next day, fresh media was added and cells were treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. The cells were stained with 1ml of 5mM MitoSOX dye. Subsequently, cells were washed 2x with PBS, resuspended in PBS, and analyzed on a FACScan. Changes in mitochondrial superoxide formation was examined by using CellQuest software and expressed as the geometric mean florescence intensity (MFI) with channel FL2.

2.5 Western Blotting

Cells were treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. The cells were scraped, washed with cold PBS and resuspended in 0.1 ml lysis buffer (5mM NaH_2PO_4, 1mM DTT, 10% glycerol, 1mM PMSF, 10μg/ml each aprotinin and leupeptin, pH 7.4) at 4°C. Extracts were centrifuged at 14,000 rpm at 4°C, and supernatants were transferred to fresh tubes. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Mississauga, Ontario, Canada). To detect E7, Ras, BCL-2, β-actin and GAPDH, 50μg of protein was prepared. Immunostaining for β-actin and GAPDH confirmed equal protein loading. Volumes of samples were added to equal volume of 4x loading dye and run in a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and stained with 0.1% Ponceau S in 5% acetic acid to ensure equal loading. Membranes were then blocked with 5% milk in TBS containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membrane was then blotted overnight at 4°C with antibody for HPV 16 E7 (1:500, Santa Cruz, Texas, U.S.A), H-Ras (1:500, Calbiochem, Darmstadt, Germany) and BCL-2 (1:1000, Sigma St-Louis, MO, U.S.A.), β-actin (1:10000, Sigma St-Louis, MO, U.S.A.) and GAPDH (1:10000, Cell Signaling Danvers, MA, U.S.A.). Membranes were washed 3 times with TBST for 10 minutes and then incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 1 hour at room temperature. Membranes were washed once again for 3 intervals of 10 minutes in TBST. The peroxidase activity was visualized by enhanced chemiluminescence (ECL; GE Healthcare Biosciences-Amersham, Little Chalfont, Buckinghamshire, United Kingdom) or chemiluminescent HRP substrate (Millipore Coorporation, Billerica, MA, U.S.A.).

2.6 Metabolic Assays

Respirometry (OCR) and the extracellular acidification rate (ECAR) of cells were measured with an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, U.S.A.). Cells were plated at 5000 cells/well and treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. The media was removed and replaced by nonbuffered Dulbecco's modified Eagle's medium containing 25 mM glucose and 2 mM glutamine. Cells were incubated in a CO2-free incubator at 37^{o}C for 1 hour to allow for temperature and pH equilibration prior to loading into the XF96 apparatus. XF assays consisted of sequential mix (3 min), pause (3 min), and measurement (6 min) cycles, allowing for determination of OCR/ECAR every 6 min. The cells were subsequently stained with crystal violet or counted to normalize the results to the quantity or number of cells of each well.

2.7 Mitochondrial Size and Number

Cells were seeded in 10 cm dishes at a density of 5 x 10^4 cells/plate. The next day, fresh media was added and cells were treated with 5μM ATO alone or in combination with 100μM trolox for 24 hours. Cells were fixed in paraffin and each sample was sliced. Intracellular pictures were taken with Transmission Electron Microscopy (TEM) at FEMR (McGill University, QC, Canada). For each condition, 6 pictures of whole cells were captured to be analyzed. The number of mitochondria and the length of the organelle were determined using ImageJ software.

2.8 Statistical Analysis

The significance of data was determined using Prism version 3.0. Analysis of variance followed by Newman-Keuls post-tests were used to determine if cell treatments produced significant changes.

CHAPTER 3: RESULTS

3.1 Introduction

Arsenic trioxide (ATO) is now used as a first-line treatment for patients suffering from acute promyelocytic leukemia (APL).[24] ATO induces cytotoxicity in both cancer and non-cancer cells by generating oxidative stress within the cell,[59] in part, due to the production of ROS[57,125]. Although ATO has been successfully in APL, dose limiting toxicities restrain its progress as an effective treatment as a single agent for other cancers. Strategies need to be developed in order to administer ATO to non-APL patients. These include using different arsenicals, such as Darinaparsin, to increase intracellular arsenic accumulation in cancer cells[126] and combining ATO with the vitamin E derivative, Trolox. In this paper, we focus on using ATO in combination with another chemical agent and how it may prove to be effective for non-APL patients. To counter the damaging effects of ATO in non-cancer cells, an antioxidant would be most suitable for protecting these cells. We previously found that trolox enhances ATO-induced apoptosis in APL cells and P388 lymphoma mouse model, while simultaneously protecting normal cells. [4,5] The objective of the current study is to understand the mechanism(s) by which trolox switches from a protective role to a potentiating role upon the oncogenic transformation of cells.

3.2 Oncogenic transformation by E6/E7 and E1A/RAS results in an imbalance in cellular homeostasis

In order to study the effects of ATO in combination with trolox, two isogenic cell line models were chosen. Wild-type mouse embryonic fibroblasts (MEF) were either transduced with oncogenes E6 and E7 or E1A and Ras. For one of the isogenic models, wild-type MEFs were also transduced with an empty vector, identified as MEF pLPC, as a control. This control was compared to MEF oncogenically transformed with E1A and Ras. Wild-type MEFs were compared to MEF oncogenically transformed with E6 and E7. One of the hallmarks of transformed cells is the acquired ability of the cells to proliferate at a higher rate than normal cells.[127] The proliferation rate of both MEF E6/7 and MEF E1A/Ras were compared to their normal counterpart. All cell lines were seeded and counted after 1, 3, and 6 days of proliferation. Both MEF E6/7 and MEF E1A/Ras cells proliferate at a higher rate than their normal cell line counterpart (Figure 1A, B). Transduction of the oncogenes and oncogenic protein expression was confirmed by blotting for either E7 or Ras antibody (Figure 1C). As shown in the western blot, MEF E6/7 overexpress E7 oncoprotein and MEF E1A/Ras overexpresses Ras protein compared to normal cells.

3.3 Trolox differentially modulates ATO toxicity in normal and oncogenically transformed cells

We have previously shown that the effect of ATO in combination with trolox is dependent on cell type: Results show trolox has a potentiating effect in transformed cells, meanwhile, it plays a

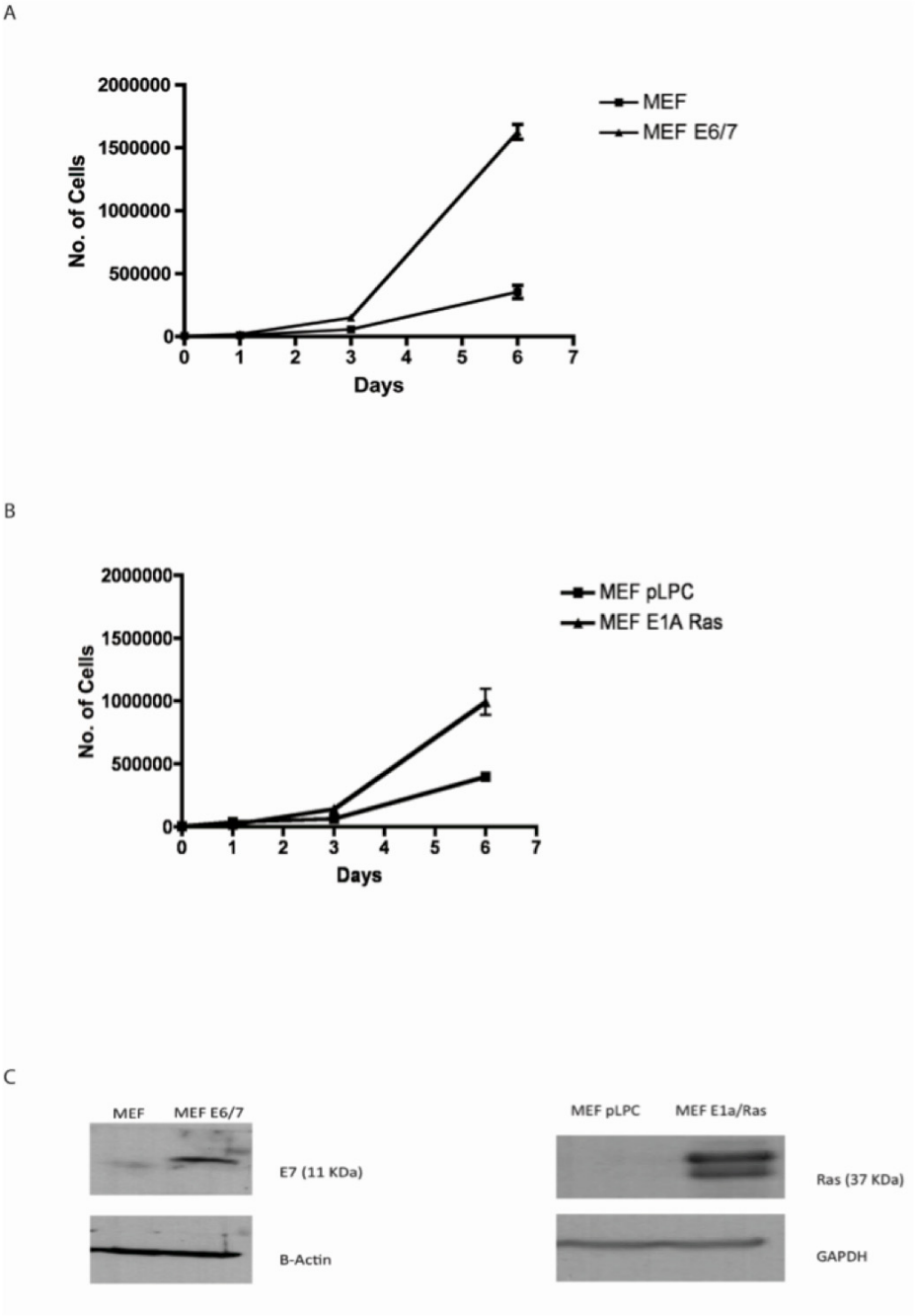
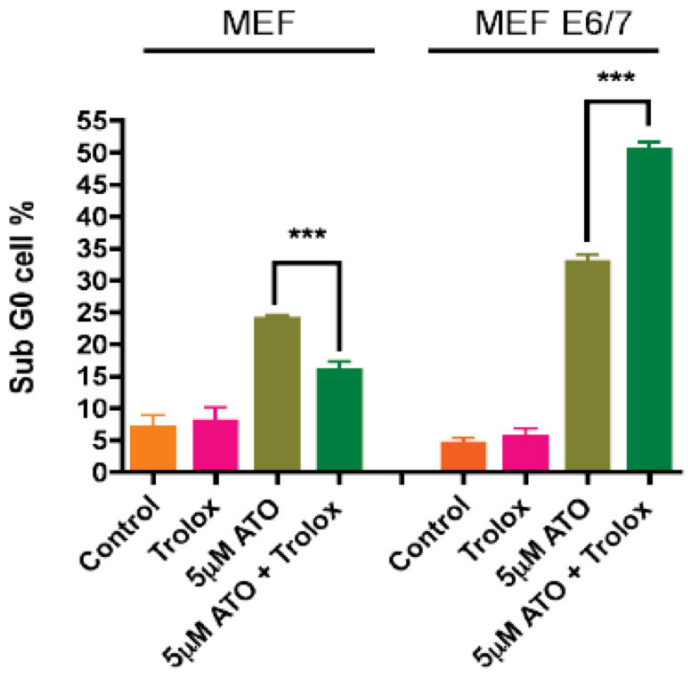


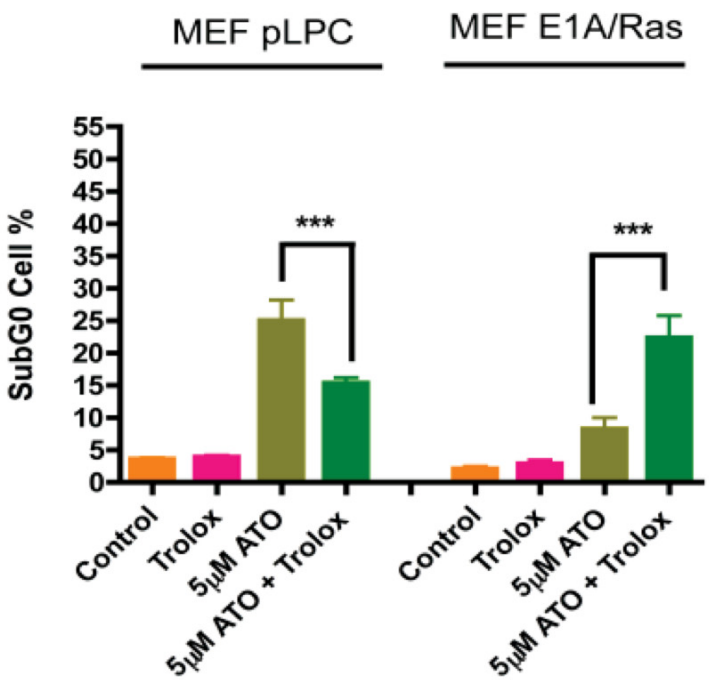
Figure 1 Oncogenically transformed cells proliferate at a higher rate than their normal counterpart. (A) Growth curve of MEF vs. MEF E6/7. (B) Growth curve of MEF pLPC vs. MEF E1A/Ras. (C) Western blots of E7 and Ras expression. B-actin and GAPDH are used as loading controls.

protective role in normal cells.[5,4] This result was repeated in both isogenic cell models defined above. Similar to the outcomes found in Diaz *et al.* 2005 and 2007, ATO-induced toxicity was differentially modulated by trolox in the normal cell lines (MEF, MEF pLPCs) versus the oncogenically transformed cell lines (MEF E6/7, MEF E1A/Ras). Cells were treated with 5μM ATO with or without 100μM trolox. Subsequently, the cells were stained with propidium iodide to determine the SubG0 cell population for each condition. When treated with ATO alone, ATO-induced apoptosis occurred in all cell types. When treated in combination with trolox in MEF E6/7 cells, the combination has increased cytotoxic effects in cancer cells but not the non-transformed cell line (Figure 2A). The difference in ATO-induced cytotoxicity was re-capitulated in the second isogenic model: MEF pLPCs and MEF E1A/Ras (Figure 2B). This result supports our previous research that trolox differentially modulates ATO-toxicity in normal and oncogenically transformed cells.[4,5] Furthermore, we examined the proliferation rate of each cell line in the conditions stated above. Cell lines were treated with either ATO alone or with trolox and counted throughout 6 days. Figure 2C shows that MEF treated with ATO in combination with trolox proliferated at a greater rate than treated with ATO alone. Contrary to MEFs, MEF E6/7 proliferated at a lesser rate when treated with ATO in combination with trolox. This correlates with the SubG0 data in Figure 2A. The trend is repeated in the MEF pLPC and MEF E1A/Ras isogenic model (Figure 2D). BCL-2 is a well-known survival marker of the cell.[47,48] In order to investigate the expression of BCL-2 protein in each condition in MEF and MEF E6/7, the cells were treated for 18h with ATO alone or in combination with trolox and the protein from these samples were then harvested and lysed. The cell lysates were blotted with BCL-2 antibody. Figure 2E shows that the expression of BCL-2 decreased in MEFs treated with

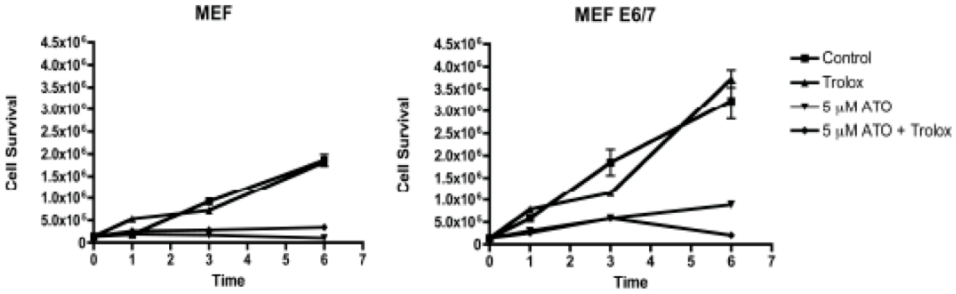
A


MEF
MEF E6/7
Sub G0 cell %
55
50
45
40
35
30
25
20
15
10
5
0
***
***
Control
Trolox
5μM ATO
5μM ATO + Trolox
Control
Trolox
5μM ATO
5μM ATO + Trolox

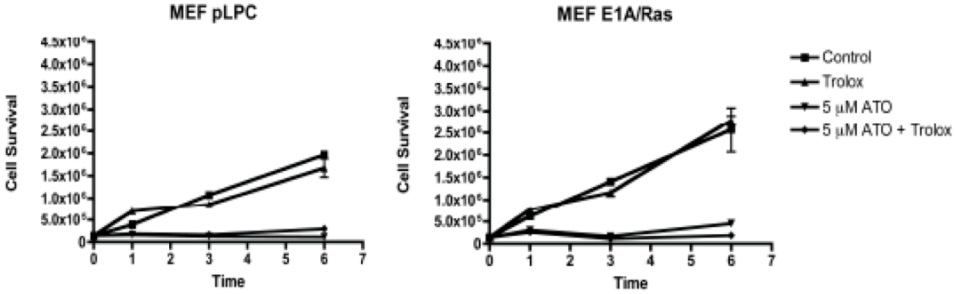
B


MEF pLPC
MEF E1A/Ras
SubG0 Cell %
55
50
45
40
35
30
25
20
15
10
5
0
***
***
Control
Trolox
5μM ATO
5μM ATO + Trolox
Control
Trolox
5μM ATO
5μM ATO + Trolox

C


MEF
MEF E6/7
Cell Survival
Time
Control
Trolox
5 μM ATO
5 μM ATO + Trolox

D


MEF pLPC
MEF E1A/Ras
Cell Survival
Time
Control
Trolox
5 μM ATO
5 μM ATO + Trolox

E

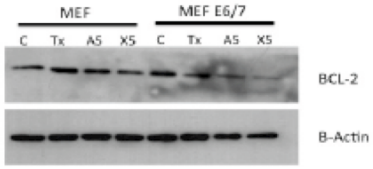

MEF
MEF E6/7
C
Tx
A5
X5
C
Tx
A5
X5
BCL-2
B-Actin

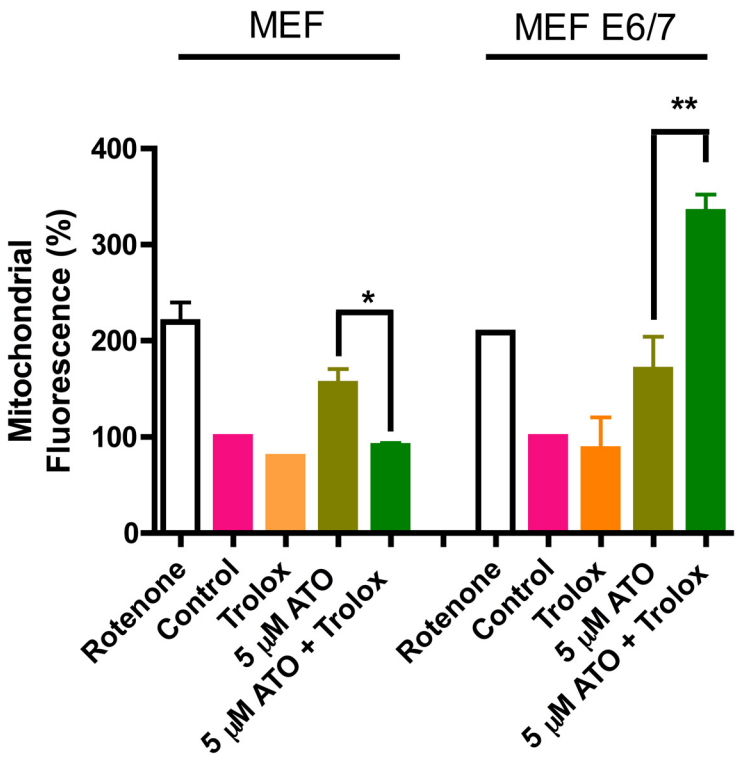
Figure 2 Transformed cells are more highly sensitive to arsenic trioxide induced cytotoxicity in combination with trolox in comparison to their wild-type counterpart. Propidium iodide stain of MEF and MEF E6/7 (A) and MEF pLPC and MEF E1A/Ras (B) after being treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. Growth curves of MEF and MEF E6/7 (C) and MEF pLPC and MEF E1A/Ras with corresponding treatment from PI. (E) BCL-2 expression by western blot.

ATO alone. However, the expression of this protein returns to normal levels when MEFs are treated with ATO in combination with trolox. In contrast, MEF E6/7 BCL-2 levels decrease in both ATO-treatment and combination treatment. The expression of the survival marker suggests that MEF cells are more likely to be protected from the harmful effects of ATO when treated in conjunction with trolox, however, MEF E6/7 cells do not express BCL-2 and so they are not rescued.

3.4 Altered mitochondrial morphology and function in response to ATO in combination with trolox.

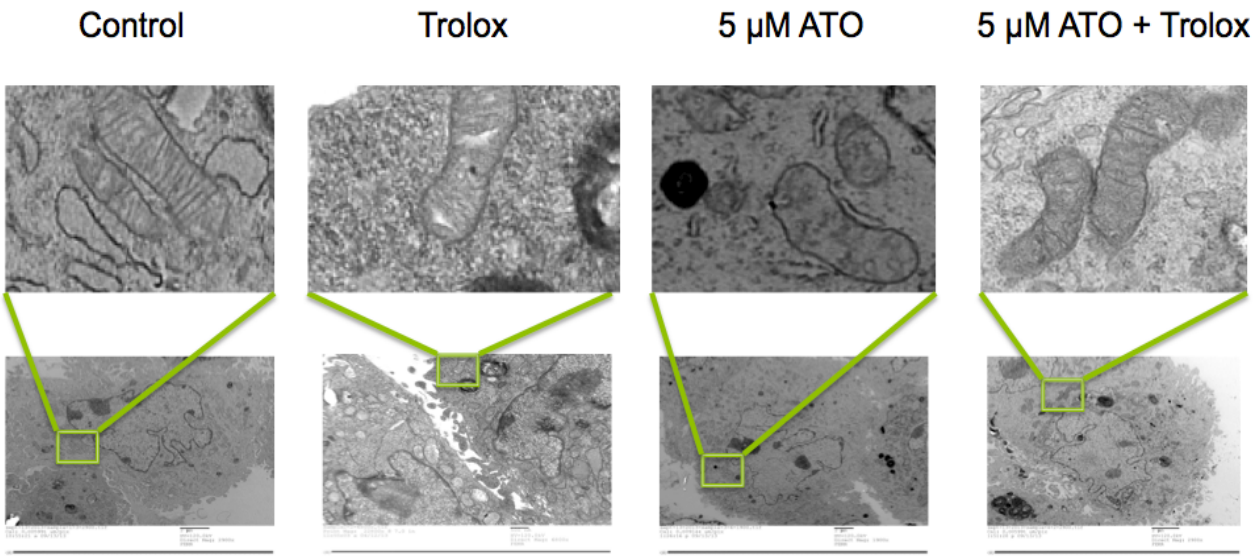
The cellular effects of ATO in combination with trolox led to the investigation of the causes of cytotoxicity within oncogenically transformed cells. We have previously shown that ATO induces cytoplasmic HO-1, a marker of oxidative stress.[5] Intracellular sources of oxidative stress include lysosomes,[128] NADPH oxidases (NOX)[129] and mitochondria[113]. We have previously concluded that both lysosomes and NOX are not significant sources of oxidative species when cells are treated with arsenic. One of the sources of oxidative stress is the formation of superoxide caused by the leak of electrons within the electron transport chain (ETC) of the mitochondria. In order to study how ATO and trolox affect the mitochondria, cells were seeded at 1.5 $x10^4$ cells/ml and treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. Oxidative stress occurring at the mitochondrial level is present in the form of mitochondrial superoxide.[92] Mitochondrial superoxide formation was measured using the MitoSOX stain. Mitochondrial superoxide formation increased in both cell lines when treated

A


MEF
MEF E6/7
Mitochondrial Fluorescence (%)
400
300
200
100
0
*
**
Rotenone
Control
Trolox
5 μM ATO
5 μM ATO + Trolox
Rotenone
Control
Trolox
5 μM ATO
5 μM ATO + Trolox

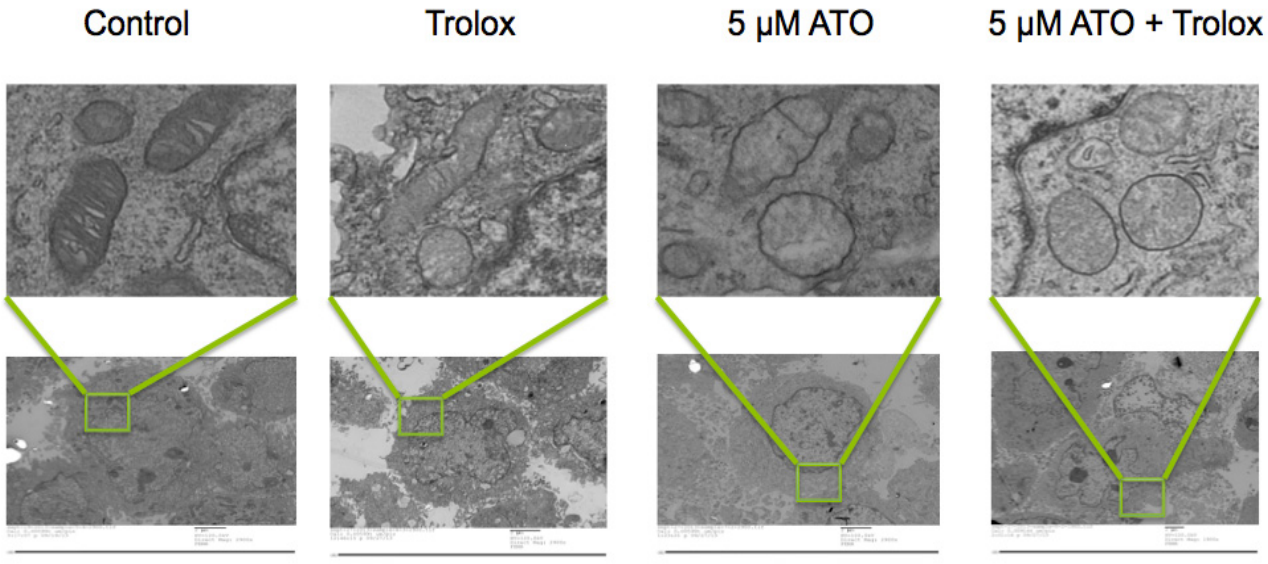
B

MEF

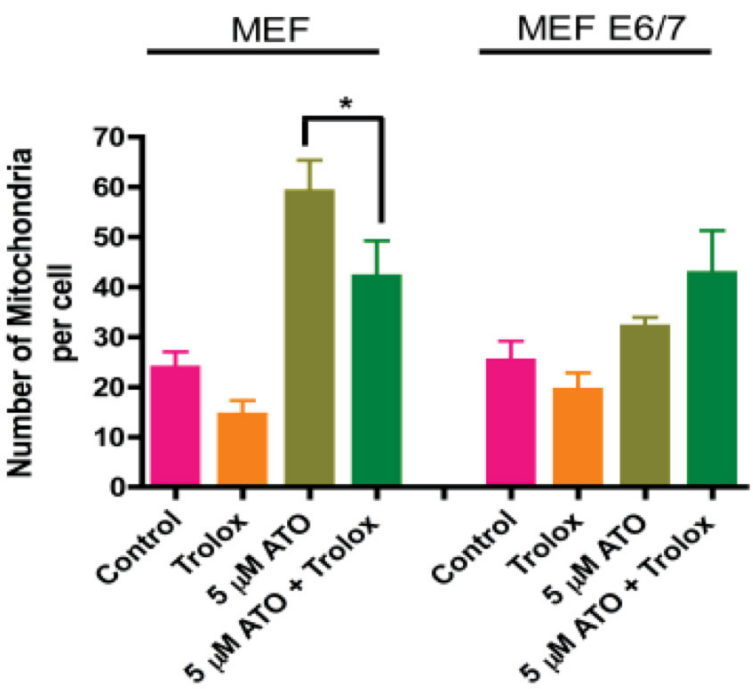


C

MEF E6/7



D


MEF
MEF E6/7
*
Number of Mitochondria per cell
70
60
50
40
30
20
10
0
Control
Trolox
5 μM ATO
5 μM ATO + Trolox
Control
Trolox
5 μM ATO
5 μM ATO + Trolox

E

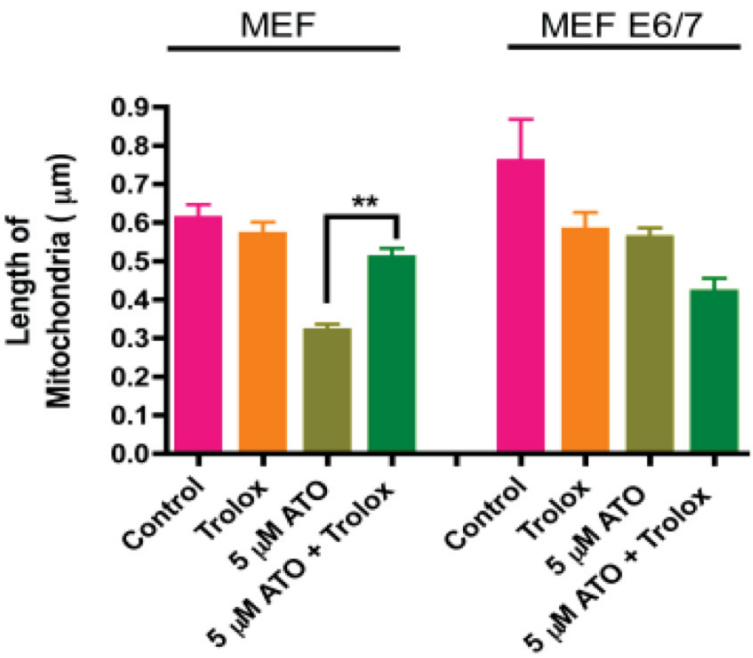

MEF
MEF E6/7
**
Length of Mitochondria (μm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Trolox
5 μM ATO
5 μM ATO + Trolox
Control
Trolox
5 μM ATO
5 μM ATO + Trolox

Figure 3 Trolox selectively enhances mitochondrial superoxide production in transformed cells and alters the morphology of these organelles. (A) Measurement of mitochondrial superoxide formation. Mitochondrial fluorescence emitted by MEF and MEF E6/7 cells were normalized to their respective controls. TEM slides of MEF (B) and MEF E6/7 (C) treated with 5μM ATO alone or in combination with 100μM trolox for 18 hours. Lengths of mitochondria (D) and number of the mitochondria (E) were measured.

with ATO (Figure 3A). When treated in combination with trolox, in comparison to ATO alone, MEF mitochondria produced less superoxide, while MEF E6/7 produced a greater amount of mitochondrial superoxide. These results demonstrate a similar trend as shown for the PI stain and suggest that trolox selectively enhances mitochondrial superoxide production in transformed cells.

The function of the mitochondria is tightly linked to their structure.[130] In order to study the physical integrity of the organelle, the cells were seeded at 5.0 x 10^4 cells/ml, treated with 5μM ATO alone or in combination with 100μM trolox for 24 hours. The samples were subsequently fixed in paraffin, sliced and observed under a transmission electron microscope (TEM). For both MEF and MEF E6/7 treated with ATO alone, the TEM reveal that the mitochondrial cristae are deteriorated (Figure 3B and C). When treated in combination with trolox, mitochondria in MEFs display long formations and tight cristae, which indicates healthy-looking mitochondria. Concurrently, mitochondria found in MEF E6/7 remained deteriorated. This suggests that trolox protects the integrity of the mitochondria in MEFs, but not in MEF E6/7. Mitochondrial responses to ATO in combination do not only affect the morphology of the organelles, the treatment may also change the number and size of the mitochondria.

Mitochondria are dynamic organelles: they can either fuse together to make long but few formations or they can adapt a fission state when stressed, which results in many but smaller mitochondria. In order to establish a modulation in the size and shape of the mitochondria, the mitochondria of six TEM slides of each condition were counted and measured. When treated with ATO alone, both MEF and MEF E6/7 adopt a fission mitochondrial profile; i.e. the

mitochondria are numerous and small in size. When treated in combination with trolox, MEF mitochondria increase in size and decrease in number, showing a mitochondrial fusion state. In contrast, MEF E6/7 mitochondria remain in a fission state when treated in combination (Figure 3D and E). These results suggest that trolox have a role in protecting the integrity of the mitochondria in MEFs and not in oncogenically transformed cells, therefore, when treated in combination with ATO, trolox differentially modulates mitochondrial function and its structure.

3.5 ATO in combination with trolox modulates mitochondrial metabolomic efficiency

There are two main pathways to generate energy in the form of ATP within the cell: glycolysis and the ETC of the mitochondria.[131] To address the role of ATO in combination with trolox in regulating the metabolism of MEF cells, we conducted a targeted metabolomic analysis by employing the Seahorse Bioscience XF Extracellular Flux Analyzer. We simultaneously measured glycolysis by assessing the extracellular acidification rate (ECAR), as well as the rate of oxidative phosphorylation by means of measuring the cellular oxygen consumption rate (OCR).[132] MEF and MEF E6/7 were plated in a 96 well plate at 5.0×10^3 cells/ml and treated with ATO alone or in combination with trolox for 18 hours. Figure 4A shows basal OCR of both cell lines. MEF treated with ATO alone have a decrease in OCR, but the OCR is restored when treated in combination with trolox. Surprisingly, MEF E6/7 basal OCR is notably higher than MEF for all conditions. However, basal OCR measured in Figure 4A does not demonstrate the efficiency of oxidative phosphorylation of the mitochondria. In order to determine the mitochondrial capability of producing ATP via aerobic respiration in both cell lines, oligomycin,

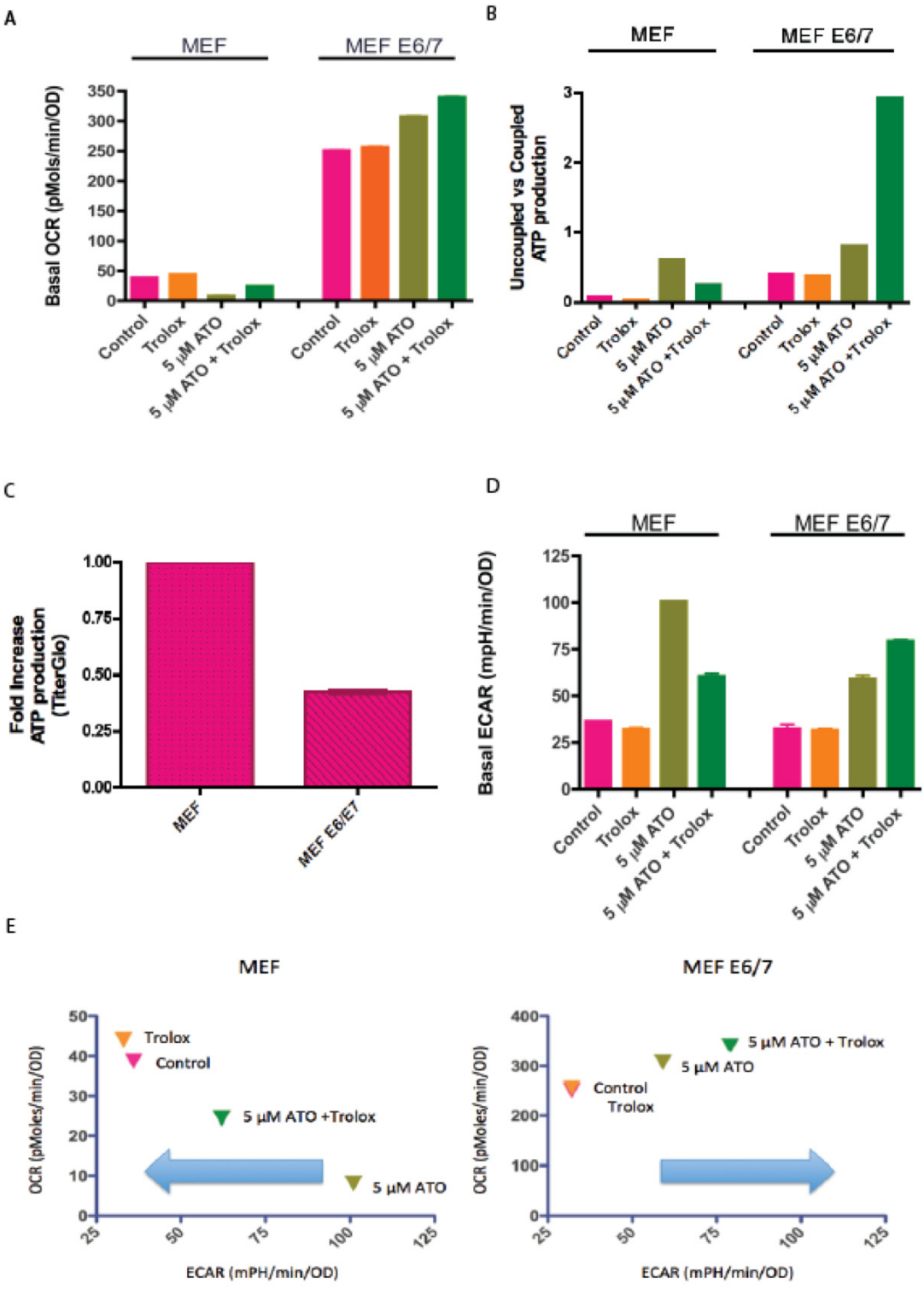


Figure 4 Mitochondrial efficiency is altered in oncogenically transformed cells. Basal OCR is shown (A) and the ratio of uncoupled versus coupled oxygen consumption was calculated for both cell lines (B) to corrected for oxygen consumption. (C) ATP production in both cell lines in control condition. (D) Basal ECAR measurements. Basal OCR and ECAR are compared (E).

which inhibits ATP-synthase, was administered to each sample. The ratio of uncoupled versus coupled oxygen consumption was calculated for both cell lines in all conditions. When the cells are administered with oligomycin, the ATP-synthase complex is inhibited and the protons leak from the mitochondria. This process creates heat within the cell and the protons are not used for ATP production.[133] When corrected for oxygen consumption driving the mitochondrial proton leak, the ratio of uncoupled vs. coupled oxygen consumption in MEF cells in control condition was less than that of the MEF E6/7, suggesting that MEF mitochondria are more efficient coupling the oxygen to ATP production than transformed cells basally (Figure 4B). MEF mitochondria efficacy decreases when treated with ATO, but is partially restored when administered in combination with trolox. In contrast, trolox potentiates MEF E6/7 ATO-induced mitochondrial inefficiency. These results suggest that MEF E6/7 are efficiently consuming oxygen, since these cells have a high basal OCR, however, the MEF E6/7 are inefficient in oxidizing substrates in order to produce energy in the form of ATP. These findings are further supported by the ATP production observed in both cell lines in control conditions. ATP production was measured using TiterGlo. Figure 4C shows that at basal levels, greater ATP production in MEF is observed when compared to MEF E6/7. Figure 4D shows basal ECAR, a measure of utilization of glycolysis, of both cell lines. When treated with ATO, both MEF and MEF E6/7 increase in basal ECAR. Therefore, ATO induces anaerobic respiration. Upon administration in combination with trolox, MEF cells' ECAR decreases and returns to baseline, however, MEF E6/7 demonstrates a further increase in ECAR in this combination treatment. Figure 4E shows the OCR results compared to the corresponding ECAR data collected from both cell lines. This analysis further demonstrates the efficacy of the mitochondria from each sample. MEF treated with ATO alone decrease in OCR and increase in ECAR. In combination with

trolox, the mitochondrial efficiency of the MEF is partially rescued since both the OCR and ECAR partially return to baseline conditions. When MEF E6/7 are administered ATO in combination with trolox, OCR and ECAR increases at a greater rate than when treated with ATO alone.

3.6 Mitochondrial spare respiratory capacity is altered in oncogenically transformed cells when treated with ATO in combination with trolox

The spare respiratory capacity of the mitochondrion is known as a marker of the organelle's ability to adapt to cellular stress.[134,135] The maximal ATP output of the mitochondria is partially controlled by the spare respiratory capacity, which was determined by inducing the release of the mitochondrial membrane potential by employing the addition of trifluorocarbonylcyanide phenylhydrazone (FCCP). Figure 5 demonstrates that mitochondria found in MEF control and trolox samples, compared to MEF E6/7, have a positive respiratory capacity. When treated with ATO, MEF mitochondria's spare respiratory capacity is depleted but it is partially restored when treated in combination with trolox. MEF E6/7 mitochondria's lack of spare respiratory capacity at baseline conditions is further potentiated by ATO and in combination with trolox.

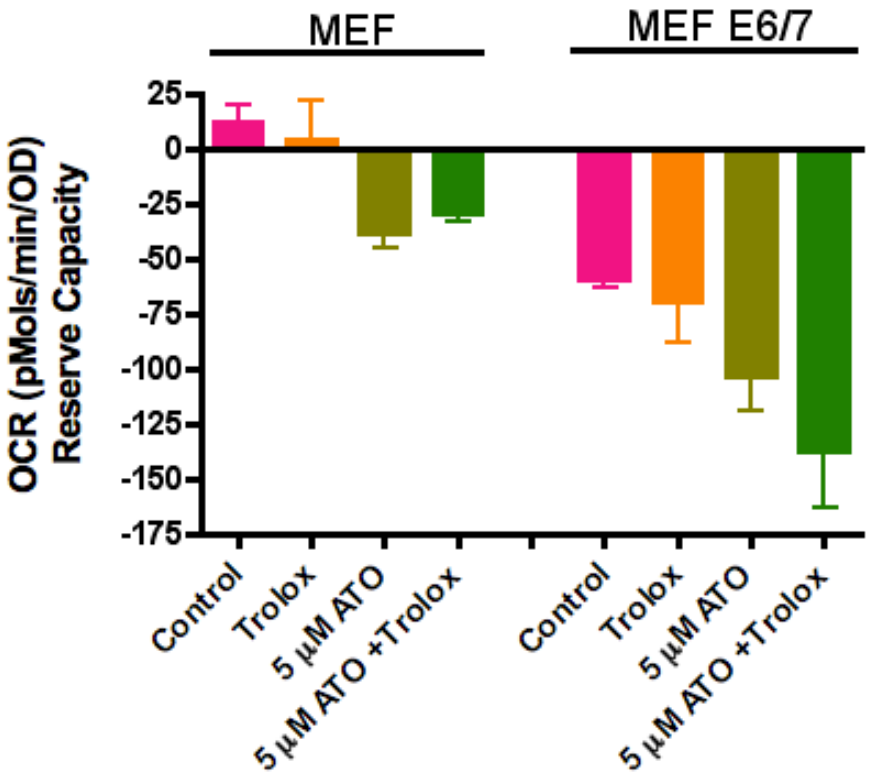


Figure 5 ATO in combination with trolox differentially modulates spare respiratory capacity. Reserve capacity in MEF cells depletes with ATO treatment. Maximal respiration is partially restored with the addition of trolox. However, when MEF E6/7 is treated with ATO in combination with trolox, spare respiratory capacity is further reduced.

3.7 Discussion

A major disadvantage of cytotoxic chemotherapy is the difficulty in selectively targeting tumor cells. Understanding the biological differences between cancer and normal cells provides a foundation for designing strategies to improve cancer therapy. Although ATO is an effective therapy in APL, its use in solid tumors is limited by the toxicity observed at concentrations required to induce apoptosis in non-APL cells. We previously found that trolox, a widely known antioxidant, enhances ATO-induced ROS and apoptosis in APL, myeloma, and breast cancer cells, while simultaneously protecting normal cells from arsenic-induced toxicity. [5,58] The objective of the current study is to understand the mechanism(s) by which trolox switches from a protective role to a potentiating role when in combination with ATO upon oncogenic transformation of cells. For this study, we compared the wild type MEF cells to MEF cells transformed with either E6/7 or E1A/Ras oncogenes. A growing body of evidence suggests that cancer cells, compared to normal cells, display increased oxidative stress, altered metabolic activity, and increased generation of reactive oxygen species (ROS).[136,137,138] We hypothesize that the predominant mechanism of action by which MEF E6/7 and E1A/Ras cells are sensitized to the actions of trolox and ATO is due to an intrinsic dysfunction of mitochondrial activity.

In both the E6/7 and E1A/Ras models, trolox enhances ATO-induced death. In contrast, trolox has a protective effect from ATO toxicity in their untransformed counterparts. When treated with ATO alone, the SubG0 population increased in all cell lines. When treated in combination with trolox, ATO-induced apoptosis decreased in untransformed cells. Meanwhile, the combination increased cytotoxicity in oncogenically transformed cells. These findings, which

also correlate to the growth curves and expression of BCL-2, support previous observations from our lab that trolox modulates ATO-induced cytotoxicity based on cell type.

ATO-induced cell death is induced in part with enhanced formation of ROS.[57] One of the intracellular sources of ROS formation occurs at the mitochondria.[115] In this study, we investigated the formation of mitochondrial superoxide when treated with arsenic in combination with trolox. Data was collected after the cells were treated and stained with MitoSOX, a stain specifically for mitochondrial superoxide. The results demonstrated that ATO induces the formation of mitochondrial superoxide in MEF and MEF E6/7. MitoSOX positive subpopulation increases in MEF E6/7 cells when subsequently treated with trolox, demonstrating that trolox further potentiates the effect of arsenic trioxide on mitochondria. However, when treated with ATO in combination with trolox, mitochondrial superoxide formation decreases in MEF cells, which suggests that trolox not only has a cytoprotective role but also a mitochondrial protective effect in wild-type cells. The trend of mitochondrial superoxide formation observed in both cell lines is similar to the trend demonstrated by the SubG0 cell population. This correlation suggests that the ROS produced by the mitochondria may contribute to the cytotoxicity resulting from ATO treatment.

The functionality of mitochondria is closely linked to their structure.[139] Previous studies have shown that enhanced fission[140] or reduced mitochondrial fusion can induce apoptotic-signaling pathways in live cells. Furthermore, promoting mitochondrial fission and network fragmentation has been associated with reduced cellular respiratory capacity and increased ROS production. [141,142,143] In contrast, upregulation of mitochondrial fusion[141] or downregulation of

fission[140] can reduce cell death. Furthermore, inhibiting mitochondrial fission also prevents fission-induced ROS.[144,145] We investigated the effect of ATO in combination with trolox on the morphology of the mitochondria found in both cell lines. Preliminary analysis of TEM images indicates that mitochondrial structural integrity in wild-type and transformed cells deteriorates when treated with ATO alone. When treated in combination with trolox, mitochondria found in MEF were restored to long, fused organelles, however, MEF E6/7 remained mostly in a mitochondrial fission state. An evident correlation is observed when the integrity of the mitochondria is compared to the mitochondrial superoxide formation in the corresponding treatment. Deteriorated cristae observed within the organelle, found in MEF E6/7 treated with ATO and in combination with trolox as well as MEF treated with ATO alone, correspond very well to an increase of mitochondrial superoxide formation. Cells with intact cristae, including MEF cells treated with ATO in combination with trolox, displayed lower levels of mitochondrial superoxide formation.

Finally, these results are supported by changes in cellular metabolism in the transformed cell lines. Alteration in cellular metabolism in cancer cells is commonly known as the Warburg effect. Warburg's theory describes that most cancer cells primarily generate energy by glycolysis via lactic acid production in the cytosol, rather than producing ATP by oxidative phosphorylation of pyruvate in the mitochondria.[146] In this study, we established measurements of glycolytic energetic pathways and aerobic metabolism of MEF cells in real time. We detected differences in both metabolic pathways in MEF and MEF E6/7. We demonstrate that ATO is a negative regulator of both anaerobic and aerobic glycolysis and cellular biosynthesis in cancer cells.

Surprisingly, MEF E6/7 has a higher basal OCR than MEF cells. This finding seems contradictory to Warburg's theory of the mitochondrial metabolism in cancer versus normal cells. However, there is evidence that there are other cancer models that do not comply with Warburg's theory. For example, oxidative phosphorylation, as well the generation of ROS, is essential for the tumorigenicity of K-Ras activated oncogenic transformation.[2] Other studies also show that oncogenic transformed cells depend on mitochondrial metabolism in order to thrive.[147,148,149] However, even though more oxygen was consumed in transformed cells, this result does not portray the efficiency of aerobic respiration. When corrected for oxygen consumption, our results revealed ATP production does vary between cell lines and based on condition. Uncoupled oxygen consumption is observed in both MEF and MEF E6/7 when treated with ATO alone. When administered in combination with trolox, the treatment restores mitochondrial efficiency in MEF and reduces proton leak from the organelle. In contrast, results show that inefficiency of MEF E6/7 mitochondria increases upon administration of combination treatment.

We then investigated the anaerobic metabolic pathway. When treated with ATO alone, both MEF and MEF E6/7 produced more lactic acid, which increased ECAR, suggesting that glycolysis seemed to be the preferred energy-producing pathway when treated with ATO alone. The increase in ECAR corresponds with a decrease in OCR in both cell types, which supports Warburg's theory that decreasing the efficiency of the mitochondria promotes anaerobic respiration. Following FCCP-injection, it was observed that ATP production is severely depressed in MEF E6/7 at baseline conditions. This suggests that mitochondria found in transformed cells are not efficient at maintaining the MMP in order to provide maximal

respiration to form ATP. The results show that ATO has a similar effect and it induces a depletion of maximal ATP production in both cell lines. Upon the addition of trolox, the MEF maximal aerobic respiration is partially restored, whereas, MEF E6/7 maximal ATP production is further depleted. When considering the ECAR data, the results observed from maximal aerobic respiration suggests that reducing the mitochondrial respiration correlates with a metabolic shift and an increase in lactate production via glycolysis. Furthermore, the integrity of the cristae, where the oxidative phosphorylation takes place, corresponds to the changes of mitochondrial metabolism. Degraded cristae, seen in MEF and MEF E6/7 treated with ATO alone, correlate with a decreased maximal respiration and thus a reduced efficiency of oxidative phosphorylation. Observations of MEF basal ECAR further support a shift in metabolomics within the cell. When the integrity of the inner mitochondrial membrane is destroyed, a greater ECAR level demonstrates an increase dependence of cellular ATP formed via glycolysis. Consequently, when MEF cells are treated with trolox in combination with ATO, the re-emergence of healthy looking mitochondria correlates with a metabolomic profile similar to control (i.e. baseline) conditions.

Further investigation is needed in order to elucidate the reason why mitochondria found in normal cells are capable of restoring their function upon the administration of trolox when combined with ATO. One potential possibility to consider is the turn over efficiency of the mitochondria within these cells. Is trolox inducing the recycling of the damaged organelles more efficiently in normal cells? One of the central effectors of mitochondrial recycling and mitochondrial biogenesis is AMP-activated protein kinase (AMPK).[150] Investigating AMPK expression and other key regulators of mitophagy would be beneficial for demonstrating the

mechanistic events, which occur upon treatment of ATO in combination with trolox. Previous studies show that activated AMPK can phosphorylate protein Unc-51-like kinase 1 (ULK1), which in turn activates autophagy pathways and induces the recycling of organelles, including the mitochondria.[151] Furthermore, it has been shown that high mitochondrial activity enhances mitochondrial degradation and renewal in normal cells. Small GTPase Rheb, a regulator of mitochondrial energetics-induced mitophagy, plays an important role in the recycling of the organelle, which prevents mitochondrial aging, and maintains the efficiency of energy production via aerobic respiration.[152] Potential experiments to investigate trolox's role in mitochondrial recycling include knocking-down either ULK1 or Rheb expression in MEF cells and to compare the effects of ATO in combination with trolox to that of oncogenically transformed cells. If knockdown MEFs are sensitive to ATO in combination with trolox and show the same metabolic profile as oncogenically transformed cells, then further investigation of trolox's implication in mitophagy pathways is necessary.

We conclude that trolox protects MEF cells from the effects of ATO by maintaining the integrity of the mitochondria and therefore maintaining the function and keeping the organelles efficient. Our work establishes that the cytotoxicity observed upon administration of ATO into the cells is partially due to the mitochondrial activity. The results show that ATO induces cristae deterioration, which leads to an increase of mitochondrial superoxide and a decrease efficiency of ATP production. The data presented here suggest that ATO negatively influences metabolic homeostasis in proliferating cells in both cell lines. With the addition of trolox, it rescues mitochondria found in normal cells and partially restores mitochondrial function. Meanwhile, trolox further potentiates the effect of ATO in oncogenic transformed cells. And so, we conclude that transformed cells are more sensitive to cytotoxicity induced by arsenic trioxide in

combination with trolox than their wild-type counterparts. We deduce that the predominant mechanism of action by which transformed cells are sensitized to the actions of trolox and ATO is due to a dysfunction of mitochondrial activity.

Taken together, the results of this thesis might provide a means to expand the therapeutic spectrum of ATO beyond APL. The long-term goal of this study is to prevent some of the clinical manifestations of ATO-related toxicity by enhancing tumor selectivity and protecting normal tissue from toxicity.

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