

2015

# $\alpha$ -TEA's Tumor Toxicity may be Attributed to its Capability of Inducing Oxidative Damage in the Endoplasmic Reticulum

Diego Mathias Barragan Echenique  
*Portland State University*

Follow this and additional works at: <https://pdxscholar.library.pdx.edu/honorsthesis>

Let us know how access to this document benefits you.

---

## Recommended Citation

Barragan Echenique, Diego Mathias, " $\alpha$ -TEA's Tumor Toxicity may be Attributed to its Capability of Inducing Oxidative Damage in the Endoplasmic Reticulum" (2015). *University Honors Theses*. Paper 124. <https://doi.org/10.15760/honors.172>

This Thesis is brought to you for free and open access. It has been accepted for inclusion in University Honors Theses by an authorized administrator of PDXScholar. Please contact us if we can make this document more accessible: [pdxscholar@pdx.edu](mailto:pdxscholar@pdx.edu).

**$\alpha$ -TEA's Tumor Toxicity may be Attributed to its Capability of Inducing Oxidative  
Damage in the Endoplasmic Reticulum**

by

Diego Mathias Barragan Echenique

An undergraduate honors thesis submitted in partial fulfillment of the

requirements for the degree of

Bachelor of Science

in

University Honors

and

Biology

Thesis Adviser

William L. Redmond, PhD

Portland State University

2015

## **Abstract**

$\alpha$ -tocopherol ether linked acetic acid ( $\alpha$ -TEA) is a Vitamin E derivative with antineoplastic and anti-metastatic properties, tumor specificity, and immunogenic characteristics. The compound is structurally similar to vitamin E, however a key difference is that it doesn't retain any antioxidant properties. Interestingly, orally administered  $\alpha$ -TEA appears to stimulate anti-tumor immunity, which, along with its anti-metastatic and antineoplastic properties, makes the molecule's mechanism of action worth investigating. Due to the loss of its anti-oxidative properties, the production of reactive oxygen species (ROS) and the cell viability of murine and human cancer cells treated with  $\alpha$ -TEA, was analyzed over time. To determine the role that ROS play in inducing cell death, tumor cells were treated with  $\alpha$ -TEA in the presence of reduced L-Glutathione (GSH), or the soluble form of vitamin E (TPGS; D- $\alpha$ -tocopheryl polyethylene glycol succinate). We also assessed the mechanism by which  $\alpha$ -TEA affected cell death by screening for the expression of Major Histocompatibility Complex (MHC) Class I and II and the non classical MHC protein CD1d, as well as that of cell surface markers associated with immunogenic cell death (ICD), such as calreticulin. Surprisingly, our data revealed that  $\alpha$ -TEA treatment induced ROS, but that cell death was only reduced when utilizing TPGS as an antioxidant, and not when GSH was present. Further analysis revealed that  $\alpha$ -TEA doesn't induce ICD in all tumor cells, but may do so in certain cell lines. Overall, our findings support the hypothesis that  $\alpha$ -TEA induces apoptosis in cancer cells through ROS build up, and that when this pathway is blocked, these ROS will initiate death through the formation of the Mitochondrial Permeability Transition Pore (MPTP), or damage to the rough Endoplasmic Reticulum.

## **Contents**

<b><u>Abstract</u></b>	<b>ii</b>
<b><u>List of Figures</u></b>	<b>v</b>
<b><u>Introduction</u></b>	<b>1</b>
<i><math>\alpha</math>-TEA's Mechanism of Action; the Current Models</i>	2
<i>ROS-induced Cell Death as <math>\alpha</math>-TEA's Mechanism of Action</i>	2
<i><math>\alpha</math>-TEA and immunogenicity</i>	4
<b><u>Materials and Methods</u></b>	<b>6</b>
<i>Cell Culture</i>	6
<i>Assessing <math>\alpha</math>-TEA's reactive oxygen species production</i>	7
<i>Assessing the Role of ROS in <math>\alpha</math>-TEA Induced Cell Death</i>	7
<i>Expression of Immunogenic Cell Death Indicator</i>	8
<i>Evaluating the STING Pathway</i>	8
<b><u>Results</u></b>	<b>10</b>
<i><math>\alpha</math>-TEA increases Reactive Oxygen Species Levels to Different Degrees in Different Cell Lines</i>	10
<i>ROS Induction and Cell Death in <math>\alpha</math>-TEA Treated Cells</i>	14
<i>Immunogenicity of <math>\alpha</math>-TEA</i>	15
<b><u>Discussion</u></b>	<b>17</b>
<i>Dose Dependent Killing Mechanism</i>	17
<i>Cell Line Dependency May be Related to <math>\alpha</math>-TEA's Location in the Cells</i>	18
<i>Death reduction by TPGS but not by GHS Reveals <math>\alpha</math>-TEA's Location of Action</i>	18
<i><math>\alpha</math>-TEA's Tumor Specificity in Relation to its Structure and the Production of ROS</i>	20
<i><math>\alpha</math>-TEA's Capabilities of Inducing Immunogenic Cell Death</i>	20

**Acknowledgements** 22

**References** 22

## **List of Figures**

<b>Figure 1</b>	<b>12</b>
<b>Figure 2</b>	<b>14</b>
<b>Figure 3</b>	<b>18</b>

## **Introduction**

Breast cancer is the most common cancer among women worldwide. Within the United States alone, it is estimated that this type of cancer is diagnosed in over 200,000 women each year of whom 40,000 will eventually succumb to the disease. Several different strategies have been explored as a means to either treat or prevent breast cancer, and there has been speculation about the supplementation of antioxidants as a possible prevention and treatment strategies for this and other types of cancers<sup>1</sup>.

Antioxidants are a plausible alternative to conventional cancer treatment because they reduce the amount of reactive oxygen species (ROS) within cells. ROS are very reactive chemical compounds with the ability to damage cell machinery and genetic material, thus potentially inducing mutations that can convert regular cells into cancerous cells<sup>2</sup>. Vitamin E ( $\alpha$ -tocopherol) was identified as a supplement with cancer prevention potential, for its anti-oxidative role in cell membranes. Consequently Vitamin E and several synthesized analogs have been tested for their efficacy to treat or prevent cancer<sup>3</sup>.

Surprisingly, two large studies revealed that vitamin E supplementation did not reduce the probability of developing cancer. Contrary to what was hypothesized, a study looking at the occurrence of prostate cancer in males with or without vitamin E supplementation found that a high dose of vitamin E supplementation actually *increased* the probability of developing prostate cancer – a finding that led to the premature termination of the study. Interestingly, a separate set of studies found that some vitamin E derivatives did possess anti-tumor properties<sup>3,4</sup>. Alpha tocopherol ether linked acetic acid ( $\alpha$ -TEA) is one of those derivatives, and oral administration of this analogue has been

reported to slow tumor progression, reduce metastasis and increase the immunogenicity of tumors in several murine cancer models<sup>5-9</sup>.  $\alpha$ -TEA is derived from vitamin E by replacing the hydroxyl group of the 6<sup>th</sup> carbon of the Chroman Head with an acetic acid residue bonded to the carbon through an ether bond. This replacement renders the molecule redox silent but gives it antineoplastic properties<sup>6,7,9,10</sup>.

### **$\alpha$ -TEA's Mechanism of Action; the Current Models**

Studies have proposed that  $\alpha$ -TEA functions by apoptosis induction through both the intrinsic and an extrinsic pathway<sup>3</sup>. Nonetheless blockade of pro-apoptotic signaling does not stop cell death of cells treated with  $\alpha$ -TEA, hinting that a second pathway operates in  $\alpha$ -TEA's mechanism of action<sup>9</sup>. A report from Li, et al, proposed that autophagocytosis was the second pathway by which  $\alpha$ -TEA killed cells. They hypothesized that autophagosomes formed in the process of  $\alpha$ -TEA-induced cell death provided an ideal source of tumor antigens for antigen presenting cells (APCs) and that consequently, these could initiate a more pronounced anti-tumor immune response<sup>9,11</sup>. While both the autophagocytosis and the apoptosis pathways may explain why  $\alpha$ -TEA induces cell death, it is unclear how these methods account for the drug's tumor specificity and anti-metastatic properties.

### **ROS-induced Cell Death as $\alpha$ -TEA's Mechanism of Action**

A model that has the potential to better explain the properties of  $\alpha$ -TEA is that of cell death induced by the buildup of ROS. Noncancerous cells maintain a homeostatic balance of ROS. These species, which are generated in abundance by the mitochondria, have been observed to act as secondary messengers for signal transduction pathways and as components of



different processes, where they play a role in normal cell function<sup>2,12</sup>. An imbalance in this homeostasis has the potential to convert normal somatic cells into malignant tumor cells through ROS-induced DNA damage of mitochondrial or nuclear DNA. Sustained DNA damage from a moderate excess of ROS may lead to mutations that render the cells metastatic. Too much of an imbalance (eg. very high levels of ROS), however, could be expected to induce cell death in both cancerous and noncancerous cells<sup>2</sup>. The mechanisms that define this process are both myriad and controversial. Some researchers propose that ROS directly induces apoptosis, while others support a mechanism characterized by the assembly of the mitochondrial permeability transition pore (MPTP). This channel is assembled after the mitochondrial depolarization occurs due to ROS and calcium ion binding, and forms a pore through which small solutes, like the reduced form of nicotinamide adenine dinucleotide (NADH) can travel through. This NADH as well as the cytosolic NADH can then be depleted by poly (ADP-ribose) polymerase (PARP) – which gets activated by ROS damaged DNA in order to repair the damage– thus halting glycolysis and stopping the cell's metabolism<sup>2</sup>.

Cancerous cells maintain a very elaborate ROS homeostasis. ROS have the potential to increase proliferation and tumorigenesis when present levels that are only moderately above those found in normal healthy cells. Many cancer cells appear to take advantage of this dynamic by maintaining an elevated level of ROS compared to non-cancerous cells. Some tumor cells keep these high levels of ROS in check by producing an abnormally high level of antioxidants, thus maintaining conditions in which proliferation is favored<sup>13</sup>. As a result, some cancerous cells may be inherently more vulnerable to ROS-induced apoptosis

or ROS-induced MPTP induced death, due to their already high baseline levels of ROS production.

While there are no studies showing a relationship between  $\alpha$ -TEA and ROS production, there are studies on structurally similar analogs, like  $\alpha$ -TOS that indicate that ROS play a pivotal role in the drugs' capabilities of inducing cell death<sup>3,14</sup>. It is likely that due to the structural similarity that  $\alpha$ -TEA and these compounds share, that  $\alpha$ -TEA also induces or increases ROS levels in cells. Being more metabolically active than normal somatic cells, cancerous cells would be affected more by high levels of ROS. Therefore, ROS-induced apoptosis may be the primary mechanism by which  $\alpha$ -TEA slows tumor progression, and ROS-induced MPTP induced death, might be a secondary pathway by which  $\alpha$ -TEA induces cell death.

### **$\alpha$ -TEA and immunogenicity**

The vitamin E derived  $\alpha$ -TEA also possesses immunogenic properties. Previous analyses of implanted 4T1 mammary carcinoma tumors in mice that were given a diet containing  $\alpha$ -TEA, have shown that there is an increased amount of CD4<sup>+</sup> and CD8<sup>+</sup> T-Cells compared to FoxP3<sup>+</sup> CD4<sup>+</sup> regulatory T cells (Tregs) within the tumor, compared to parallel tumors in untreated mice. Additional studies demonstrated that the cytokine pool inside of the tumor of  $\alpha$ -TEA treated mice, favors a Th1 (IL-2<sup>+</sup>/IFN-g<sup>+</sup>) response.<sup>8</sup> Li et al argued that vesicles that were the result of  $\alpha$ -TEA triggered autophagy explained this observation. However, there are likely alternative mechanisms by which  $\alpha$ -TEA may elicit an immunogenic response.

It has been reported that the extracellular expression of calreticulin, HSP90, HSP70, HSP60, and secretion of HMGB1 by pre-apoptotic cells are important in eliciting an anti-tumor immune response. These proteins, when found in the extracellular matrix, interact with dendritic cells or serve as direct pro-inflammatory signals that help generate more robust adaptive and innate immune responses<sup>15</sup>. Studies looking at the translocation of calreticulin from the endoplasmic reticulum (ER) to the external leaflet of the lipid bilayer suggested that ROS were required for this process to occur<sup>16</sup>. If  $\alpha$ -TEA does in fact increase cellular level of ROS, it may boost immune function through the extracellular expression of calreticulin. Furthermore, high levels of ROS may cause enough damage to compromise intracellular compartments and the lipid bilayer. If this is the case, then the proteins could leak out to the extracellular matrix and provide a signal in a more necrosis-like fashion.

A different way in which  $\alpha$ -TEA may induce immunogenic cell death may be through the up-regulation of MHC receptors, in order to increase antigen presentation to cytotoxic T lymphocytes (CTLs). Although there is no clear mechanism as to how  $\alpha$ -TEA could cause this, it is a way in which tumor cells may become more immunogenic.

A more plausible pathway that may explain the  $\alpha$ -TEA's immunogenic behavior is that of type I interferon (IFN- $\alpha$ , IFN- $\beta$ ) secretion. ROS damaged mitochondria can lead to the release of mitochondrial DNA into the cytoplasm. Once outside the mitochondria double stranded DNA interacts with STING also known as Stimulator of Interferon Genes. STING is involved in mediating the production of interferons in response to bacterial or viral DNA<sup>17</sup>. It recognizes cyclic dinucleotides (CDNs) that are synthesized by cyclic GMP/AMP synthases when they encounter dsDNA or by CDNs of bacterial origin<sup>18</sup>. When STING is

activated, it initiates a pathway that culminates in the transcription of genes to produce type I interferons. After detection of CDNs, STING phosphorylates TANK binding protein kinase 1 (TBK1) a serine threonine kinase that later phosphorylates Interferon Regulation Factor 3 (IRF3), which will form a dimer and enter the nucleus as a transcription factor for the production of type I interferons and various chemokines. These have a direct impact on development of certain leukocytes and thus an impact in innate and adaptive immune function.

ROS may explain many of the phenomena triggered by  $\alpha$ -TEA. ROS may provide an explanation for its antitumor and anti-metastatic properties and it may link  $\alpha$ -TEA induced cell death with its observable immunogenic qualities. In this study we focus on determining whether  $\alpha$ -TEA effectively increases ROS levels in several murine and human breast cancer cell lines. Furthermore, we analyze the relationship between ROS production and cell death, and determine the nature of  $\alpha$ -TEA's immunogenicity.

## **Materials and Methods**

### **Cell Culture**

Murine 4T1 and TUBO and human MDA-MB 231 and MCF7 breast cancer cell lines were cultured in RPMI with 10% FBS (Fetal Bovine Serum), 1% Sodium Pyruvate, 1% NEAA (Non-Essential Amino Acids) solution, 1% HEPES and 1% Pen/Strep/ Glut (penicillin, streptomycin, and glutamine) solution.

### **Assessing $\alpha$ -TEA's reactive oxygen species production**

The abovementioned cell lines were plated onto flat bottom 96-well plates at 25% confluence and allowed to grow to confluency. Subsequently, the cells were administered treatments of 0, 30-, 60-, 120-, or a 240  $\mu$ M  $\alpha$ -TEA in groups of 6 wells. The  $\alpha$ -TEA utilized through all experiments was conjugated to a lysine salt in order to increase its solubility. The lysine component did increase the solubility of the compound but did not change the properties of the drug. Additionally H<sub>2</sub>-DCFDA [2', 7'-dichlorodihydrofluorescein diacetate, Invitrogen] (Ex/Em: ~492–495/517–527 nm)–a molecule that produces a fluorescent product after it reduces hydrogen peroxide– at a concentration of 40  $\mu$ M was added to a subset of the cells to detect the changes in the levels of reactive oxygen species over time. A different set of cells were administered the same  $\alpha$ -TEA treatments, but also received propidium iodide (PI) [1:40 BD Biosciences] Ex/Em: ~530/620 nm) in order to track the viability of the cells over time. PI fluoresces upon binding double stranded DNA or dsRNA, however, it is membrane impermeable and thus will only fluoresce when cells are dead or if their membranes are compromised. The fluorescence of these compounds, successively, was measured over the course of two days at different time intervals utilizing a fluorometer [Modulus Microplate Reader Turner Biosystems].

### **Assessing the Role of ROS in $\alpha$ -TEA Induced Cell Death**

4T1 cells in 96-well flat bottom plates were given  $\alpha$ -TEA in the absence or the presence of reduced L-Glutathione (GHS), or the presence of  $\alpha$ -tocopheryl polyethylene glycol succinate (TPGS), and the levels of ROS and the viability of cells were measured by fluorescence over time. GHS acts as a reducing agent for hydrogen peroxide within the mitochondrial matrix in the presence of glutathione peroxidase. It was utilized to reduce the levels of ROS in the

cells of different treatment groups. TPGS also serves as an antioxidant; this compound is synthesized by conjugating  $\alpha$ -tocopherol to a polyethylene glycol (PEG) by an ester bond to the chroman head. Inside of the cell, the bond is enzymatically cleaved and  $\alpha$ -tocopherol is delivered to the membranes in the cell where it protects lipids from peroxidation.

Essentially, it is a soluble method of delivering vitamin E to cells<sup>19</sup>. The cells were divided into treatment groups that received none or 60  $\mu$ M  $\alpha$ -TEA the presence or absence of 20-, 10-, 5-, 2.5-, 1.25, 0.625 mM GSH respectively. In a separate experiment, 4T1 cells were administered 30, 60, 120, or 240  $\mu$ M  $\alpha$ -TEA in the presence or the absence of TPGS.

### **Expression of Immunogenic Cell Death Indicator**

To measure the immunogenicity of  $\alpha$ -TEA in vitro, the translocation of calreticulin, HSP 60, HSP 90, and HSP70 to the external leaflet of the plasma membrane, as well as the expression of MHC I, MHC II, and CD1d on the surface of 4T1 and TUBO cells was surveyed. The cells were plated on to a 96well plate and were treated with and 5nM IFN $\gamma$  as a positive control of MHC II and MHC II expression, 0  $\mu$ M  $\alpha$ -TEA as a negative control, or 30  $\mu$ M  $\alpha$ -TEA for 24 hours.

The level of surface expression of these proteins was measured by coating the treated cells with fluorophore-conjugated antibodies specific for the respective proteins. Subsequently the cells were washed and their fluorescence scanned utilizing a Licor Odyssey infrared fluorescence scanner.

### **Evaluating the STING Pathway**

To evaluate whether the STING pathway is at play in  $\alpha$ -TEA treated cells, the presence of P-TBK, P-IRF3, and STING was analyzed through western blotting. 4T1 mammary carcinoma

cells and RAW 264.7 macrophages were cultured until confluent. Subsequently, 4T1 cells were treated with 30  $\mu$ M  $\alpha$ -TEA or left untreated for 24 hours. Similarly, RAW 264.7 cells were then either treated with 1  $\mu$ g/ml Lipopolysaccharide (LPS) for 4 hours, Imiquimod for 24 hours, or left untreated for 24 hours to serve as controls. LPS stimulates the production of type I interferons in macrophages by activating Toll Like Receptors (TLR), similarly Imiquimod interacts with intracellular TLRs and induces the production of interferon  $\alpha$  (INF $\alpha$ ), making macrophages pulsed with this compound an appropriate control<sup>20,21</sup>.

After treatment the cells were lysed in a 1% SDS (w/v) solution in H<sub>2</sub>O. The lysates were filtered through 5  $\mu$ M syringe filters (PALL) to filter out DNA debris and insoluble particulates. The protein concentrations were quantified by a BCA assay (Life Technologies), following manufacturer protocols. After equalizing the concentrations, the proteins were denatured in Laemmli buffer at 96°C and then separated through SDS-PAGE gel electrophoresis utilizing Mini Protean TGX Precast Gels (Bio-Rad) loading 10  $\mu$ l of the sample into each well. Following separation, the proteins were transferred to a nitrocellulose membrane, which was blocked with Odyssey Blocking Buffer (LiCor). Different membranes were then incubated with Rabbit monoclonal antibodies specific for p-TBK1, p-IRF3 and STING (Cell Signaling) respectively, at a concentration of 1:1000 overnight at 4°C. After washing four times for duration of five minutes with 0.1% tween-20 in PBS, the membranes were incubated with Fluorophore-conjugated Goat  $\alpha$ -Rabbit polyclonal antibodies (LiCor) for 2 hours at room temperature in the dark. The membranes were washed as before and imaged utilizing a LiCor Odyssey infrared scanner. After imaging the membranes, they incubated for 20 min in stripping buffer (Odyssey) at room temperature and then, after washing, the membrane previously blotted for P-TBK, and the

membrane previously blotted for P-IRF 3 were incubated overnight at 4°C with TBK, and IRF3 Rabbit anti-mouse monoclonal antibodies (Cell Signaling), respectively. Following the incubation, the membranes were washed and incubated with secondary antibody as previously described, and then imaged in a LiCor Infrared scanner.

## **Statistical Analyses**

Differences between groups of individual experiments in time course assays were analyzed through a two way ANOVA with Tukey HSD test. Differences in variance between groups in experiments with only one time point were evaluated through simple one way ANOVA with Tukey HSD test. All values with  $P < 0.05$  were regarded as statistically significant, and all statistical analyses were done using Prism 6 graphing software (GraphPad).

## **Results**

### **$\alpha$ -TEA increases Reactive Oxygen Species Levels to Different Degrees in Different Cell Lines**

4T1, TUBO, MCF-7, and MDA-MB-231 were treated in the presence or absence of  $\alpha$ -TEA and the levels of ROS and cell viability were measured over the course of two days. The graphs were normalized by subtracting the basal ROS production rates of cells in media (without  $\alpha$ -TEA treatment) from the ROS and cell death levels of cells that received treatment. All of the listed cell lines responded to  $\alpha$ -TEA similarly with respect to the rate of cell death. In each cell line, with exception of MDA-MB-231, the number of dead cells was dependent on the dosage of  $\alpha$ -TEA, with more deaths occurring at a concentration of 240  $\mu$ M  $\alpha$ -TEA and each lower dose killing off a lower number of cells. MDA-MB-231 followed



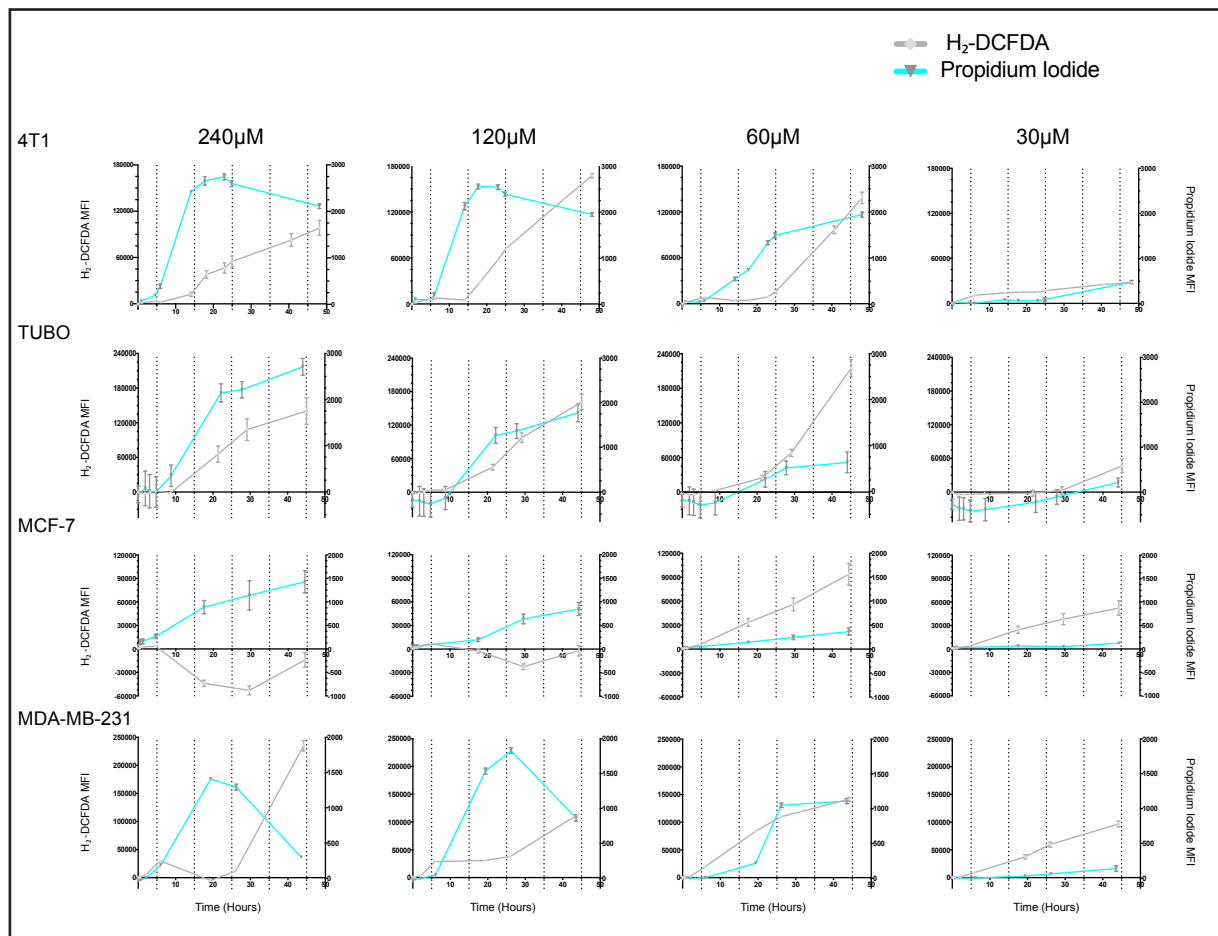
the same trend with the exception that the 120  $\mu\text{M}$  concentration of  $\alpha\text{-TEA}$  was the one that killed the most cells.

While the death rates were similar among cell lines, the production of ROS varied from cell line to cell line. In the murine 4T1 mammary carcinoma, the highest ROS levels were reached at a concentration of 120  $\mu\text{M}$  followed by the 60  $\mu\text{M}$  dosage, the 240  $\mu\text{M}$  dosage, and ending with the 30  $\mu\text{M}$   $\alpha\text{-TEA}$  dosage (**Figure 1**). The total amount of ROS and the rates at which the ROS levels rose varied among the different cell lines. While at the 240  $\mu\text{M}$  concentration the levels of ROS started rising after five hours, they did not start to pick up until 15 hours and 25 hours after treatment for the 120  $\mu\text{M}$  and 60  $\mu\text{M}$  dosages, respectively. The 30  $\mu\text{M}$  concentration experienced a slight increase at 5 hours, yet the rate at which ROS were generated did not differ much from the control, showing that it was not very efficacious at increasing ROS levels. The rates at which the ROS levels in the cells treated with a higher  $\alpha\text{-TEA}$  concentration, on the other hand, increased in a linear manner over time after they began to rise, with the steepest slope occurring at the 120  $\mu\text{M}$  concentration.

These data also indicate that cell death preceded a rise in ROS levels in all but the 30  $\mu\text{M}$   $\alpha\text{-TEA}$  concentration treatment group, where little cell death occurred.

TUBO cells interacted differently with  $\alpha\text{-TEA}$  than did the 4T1 cell line. The ROS levels began to rise after ten hours for all concentrations except the 30  $\mu\text{M}$ . However the levels of ROS were not dose dependent as was expected. The highest amounts of ROS were produced in cells treated with 60  $\mu\text{M}$   $\alpha\text{-TEA}$  whilst the two highest concentrations exhibited similar levels of reactive oxygen species and the lowest concentration barely

exceeded the control by the last time point. Interestingly, for all, but the highest  $\alpha$ -TEA dosage treatment group, cells began perishing right around the time that ROS levels began augmenting, or slightly after.



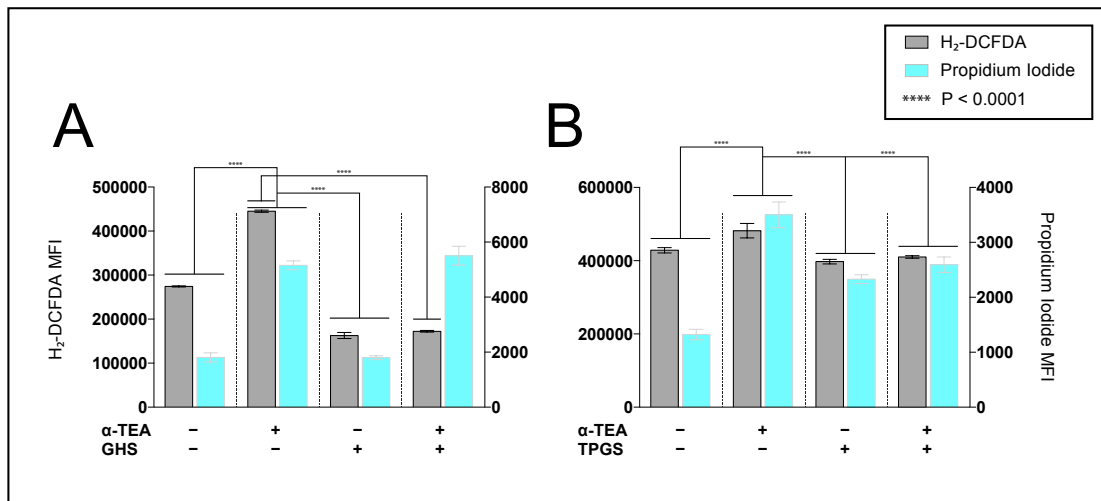
**Figure 1:** The ROS species levels and the cell viability of 4T1 and TUBO (murine) and the MCF-7 and MD-231 (human) breast cancer cell lines. Graphs for the same cell line are displayed horizontally and graphs for the same  $\alpha$ -TEA treatment concentration are displayed vertically. The graphs portray the difference in Mean Fluorescence Intensity (MFI) of 2', 7'-dichlorofluorescein (DCF) between treatment groups and the control group in left Y-axis and the difference in MFI of PI between treatment groups and the control group on the right Y-axis. In the cell, H<sub>2</sub>-DCFDA is cleaved by esterases and then reacts with ROS to generate DCF, which fluoresces when excited with 492-495nm wavelengths. Therefore its fluorescence is proportional to the number of ROS inside of cells. Similarly, PI binds to dsDNA when the cell membrane is compromised and fluoresces when excited with 530nm wavelengths, therefore its fluorescence intensity is proportional to the number of dead cells. The graphs depict the mean  $\pm$  SEM of sextuplicate samples from 1 of 2 representative experiments.

The most surprising result came from the MCF-7 cell lines, which had ROS levels at and below the basal ROS production rates at the 120  $\mu\text{M}$  and 240  $\mu\text{M}$  concentrations of  $\alpha\text{-TEA}$  respectively, but had significantly higher levels of ROS at the 30  $\mu\text{M}$  and 60  $\mu\text{M}$  concentrations. As was the trend with the TUBO cell line, the death rate of MCF-7 cell was dose dependent, but did not correlate with ROS species levels for any but the 60  $\mu\text{M}$  concentration in which cells began dying after a significant increase in ROS levels. In addition, at the concentrations in which ROS levels augmented, the rise in ROS began at an earlier time point than in the murine cell lines – roughly 5 hours after treatment.

Likewise ROS levels between the control and the treatment group commenced to diverge much sooner in MDA-MB-231 cells at all concentrations except the highest one. Although the highest levels of reactive oxygen species were produced by the highest concentration at the latest time point, this concentration didn't significantly diverge from the control until 25 hours after the experiment began. The second highest concentration induced a rise in ROS early on, but the ROS levels plateaued and commenced to pick back up 25 hours after treatment first began. In both these instances, cell death occurred sooner than the production of ROS, which could have been the cause of the phenomenon. The levels of ROS at the lower concentrations, however, rose steadily over time. At the 60  $\mu\text{M}$   $\alpha\text{-TEA}$  concentration, cell death occurred after ROS levels increased by a significant amount, yet at the 30  $\mu\text{M}$  concentration, no significant shift in death rate was visible, perhaps because the ROS levels during this time period were as high as they were when the death rate started to rise in cells treated with the 60  $\mu\text{M}$  concentration.

## ROS Induction and Cell Death in $\alpha$ -TEA Treated Cells

The levels of reactive oxygen species were reduced by the presence of either antioxidant (TPGS or GSH). However the viability of the cells was dependent on the identity of the antioxidant. While those cells treated with different dosages of GHS showed no decrease in cell death, in fact showed an increased amount of death at the higher GHS concentrations in the absence of ROS (data not shown), the cells treated with TPGS not only experienced a reduction of ROS, but also experienced a decrease in cell death. While TPGS alone caused death on its own, and  $\alpha$ -TEA alone caused significantly more cell death than TPGS alone, when they were combined, they caused as much death as with TPGS alone, hinting that the death was only caused by the apparent slight toxicity of TPGS (**Figure 2**).



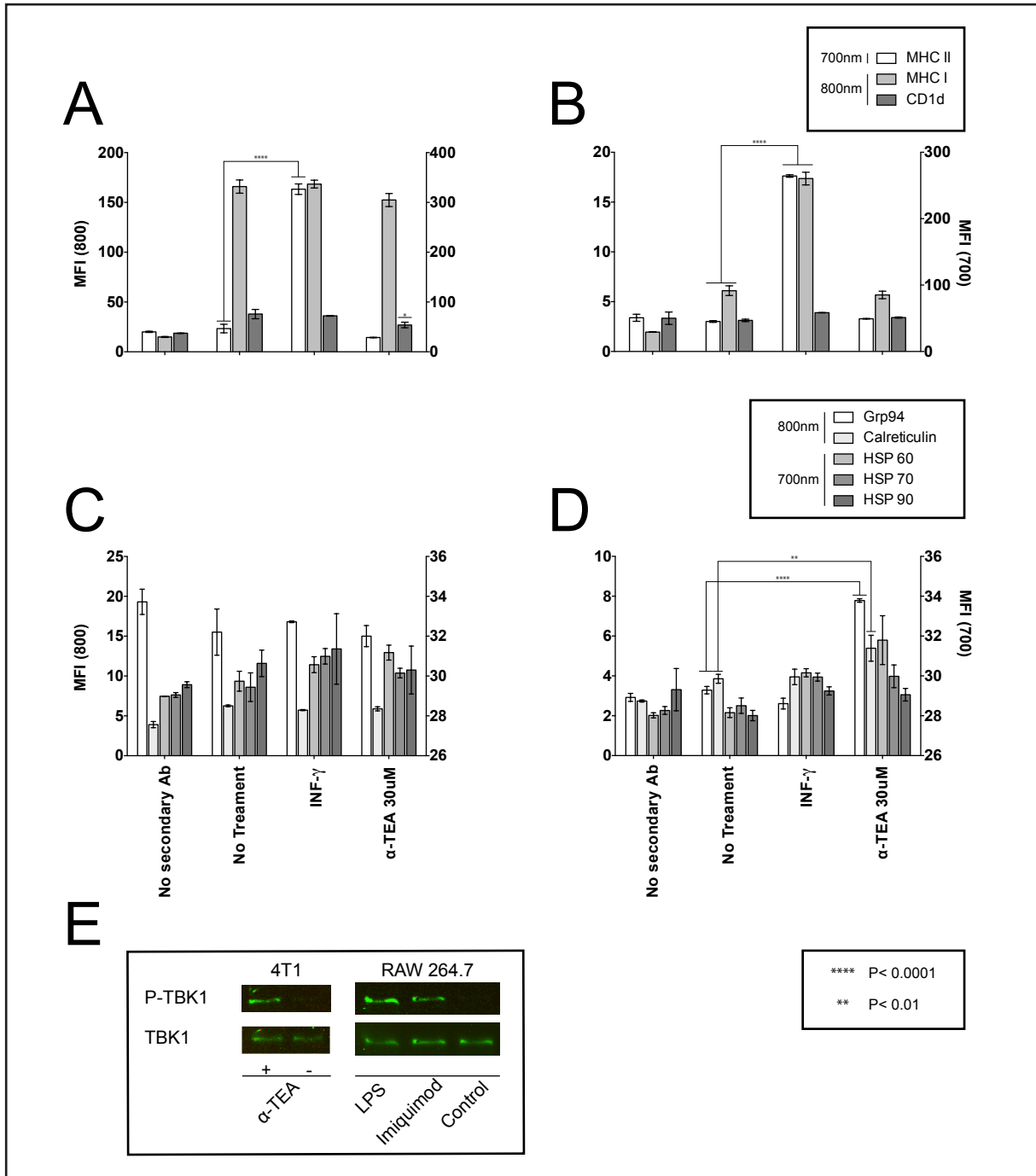
**Figure 2:** Reduced L-Glutathione (GHS) and D- $\alpha$  tocopheryl polyethylene glycol 1000 succinate (TPGS) reduce the amount of ROS in 4T1 cells treated with  $\alpha$ -TEA, but only TPGS diminishes  $\alpha$ -TEA's ability to reduce cell death. A. The ROS levels and cell death levels after approximately 45 hours, as measured by fluorescence of DCF and PI respectively, in the presence of  $\alpha$ -TEA, GSH, both, or none. B. The levels of ROS and cell death levels of cells treated with  $\alpha$ -TEA, TPGS, both, or none after approximately 45 hours. Values that are significantly different ( $P < 0.0001$ ) from those of the cells treated with  $\alpha$ -TEA only are marked by "\*\*\*\*." Graphs depict the mean  $\pm$  SEM of quadruplicate samples from 1 of 2 representative experiments.

### **Immunogenicity of $\alpha$ -TEA**

Immune fluorescence staining of immunogenic indicators revealed that  $\alpha$ -TEA's immunogenicity varied between 4T1 and TUBO cell lines. In 4T1,  $\alpha$ -TEA didn't seem to have an immunogenic effect via the up-regulation of MHC I, MHC II, or CD1d nor through the extracellular expression of HSP 60, HSP 70, HSP 90, Grp94, or calreticulin. In fact, there were statistically significantly lower levels of MHC I and CD1d expression in 4T1 cells treated with  $\alpha$ -TEA. TUBO cells, likewise, did not show an increase in MHC I or MHC II expression. Surprisingly though, they did have significantly higher expression of Grp 94 ( $P < 0.0001$ ) as well as of calreticulin ( $P < 0.01$ ), when compared to controls.

Given that  $\alpha$ -TEA has been observed to increase immunogenicity of 4T1 tumors in vivo, we analyzed whether this cell line activated the STING pathway through western blotting.

**Figure 3** only shows the results for the blots of p-TBK, and TBK 1, since blots for STING, P-IRF3, and IRF3, were all negative. Results show that only  $\alpha$ -TEA treated 4T1 cell lines express P-TBK1 as does the control cell line when treated with LPS or with Imiquimod. The untreated groups, however only expressed unphosphorylated TBK1.



**Figure 3:**  $\alpha$ -TEA's immunogenicity does not come from immunogenic cell death or from increased expression of major histocompatibility complexes in 4T1 cells, but might come from the activation of the STING pathway.  $\alpha$ -TEA may induce immunogenic cell death in TUBO cells. A & B. The expression of MHC I and MHC II of (A.) 4T1 cells and (B.) TUBO cells treated with either  $\alpha$ -TEA, INF- $\gamma$  as a positive control, or without treatment. C & D. The extracellular expression of HSP 60, HSP 70, HSP 90, Grp94, and calreticulin in (C.) 4T1 and (D.) TUBO cells treated with  $\alpha$ -TEA, INF- $\gamma$ , or no treatment at all. E. The expression of P-TBK1 and TBK1 in 4T1 cells treated with  $\alpha$ -TEA for 24 hours. The graphs depict the mean  $\pm$  SEM of duplicate samples from 1 of 2 representative experiments.

## **Discussion**

### **Dose Dependent Killing Mechanism**

Due to its high degree of structural similarity to Vitamin E, it is very likely that  $\alpha$ -TEA is located in the same cellular, sub-cellular, and extracellular compartments as the vitamin. Vitamin E is found attached to lipoproteins, within the lipid membrane, and within membranes of sub-cellular compartments. Vitamin E concentration is highest in the microsomal, lysosomal, and mitochondrial fractions of lysed cells<sup>22,23</sup>. Inside of the mitochondria, vitamin E is thought to prevent the peroxidation of lipids and it is thought to enter into a cycle with co-Enzyme Q, in which  $\alpha$ -Tocopherol will accept a free radical and co-enzyme Q will reset it, allowing it to accept a new radical<sup>23</sup>. The distribution, as well as the dynamic that vitamin E has in the mitochondria and other organelles where ROS are present, helps explain the trends that are observed in the data.

At the higher  $\alpha$ -TEA concentrations, cells die much faster, even before the levels of ROS begin to rise. At the lower concentrations, however, the levels of ROS start to increase at a higher rate than the rate of cell death. The observation seems to indicate different mechanisms of cell death dependent on the concentration of the drug. At higher concentrations it is quite likely that the  $\alpha$ -TEA-lysine salt conjugate may disrupt the membrane properties and compromise the cell membrane before it has a chance to induce oxidative stress. At this concentration, any cell death occurring through ROS would be masked by the consequences of an imbalanced membrane composition. At the lower concentrations, nonetheless,  $\alpha$ -TEA may impact the oxidative stress in cells without causing an adverse effect on the membrane composition.  $\alpha$ -TEA would then replace

vitamin E in the membranes of cellular sub compartments, where it would disrupt the existing ROS homeostasis because it cannot act as an antioxidant. Subsequently, levels of ROS would rise and ROS would cause lipid peroxidation and protein damage until enough damage would cause apoptosis or other forms of death. The physiological concentration in mice is around 3 ug/ml, therefore the latter mechanism would more closely resemble what is occurring in vivo.

### **Cell Line Dependency May be Related to $\alpha$ -TEA's Location in the Cells**

As demonstrated in **Figure 1**,  $\alpha$ -TEA interacted differently with different cell lines. In particular, the rate of ROS production, the time at which ROS began to rise, and the overall rate of cell death varied substantially between cancer cell lines. This may be attributed to the chemical nature of the drug. As stated previously, the similarity to vitamin E indicates that the compound may predominantly be present in membranes. Different tumor cell lines are likely to have different membrane compositions and different membrane fluidity, which might determine the amount of  $\alpha$ -TEA that will incorporate into the membranes of each cell line. Furthermore the ratios of  $\alpha$ -TEA in the organelles of different cell lines will also vary and impact the efficacy that the drug has on suppressing each tumor cell line. To assess whether this difference can be attributed to varying membrane compositions among the cell lines, the ratios between different lipid components would need to be evaluated as well as the concentration of  $\alpha$ -TEA in different fractions of the cells.

### **Death reduction by TPGS but not by GHS Reveals $\alpha$ -TEA's Location of Action**

When the cells were treated with  $\alpha$ -TEA and either one of two different types of antioxidants—GHS or TPGS — their production of ROS was lowered. Nonetheless, TPGS



reduced  $\alpha$ -TEA's ability to induce cell death, while GSH had no such effect on cell death. This surprising finding provides an important insight into where  $\alpha$ -TEA may be acting. TPGS is also a vitamin E analog that is much more water-soluble, yet retains antioxidant activity after it is cleaved by cellular esterases, causing it to deliver  $\alpha$ -tocopherol to the membranes. This compound is used as a vitamin E supplement and it is also utilized to facilitate the delivery of hydrophobic drugs to cells in the body through the plasma membrane. Thus, just like vitamin E, it would be found at its highest quantities in the microsomal, mitochondrial, and lysosomal fractions of lysed cells. The microsomal region is mostly formed by remnants of the rough endoplasmic reticulum (rER), smooth endoplasmic reticulum (sER) and cellular vesicles<sup>24</sup>. Therefore it is conceivable that TPGS and  $\alpha$ -TEA would accumulate at this location. It just happens that GSH is found ubiquitously throughout the cell, with the only exception being the lumen of the rER<sup>25</sup>. While the presence of GSH throughout the cell would protect from the activity of ROS induced by  $\alpha$ -TEA, GSH would not be able to protect the cell from the damage that increased amounts of ROS induced by  $\alpha$ -TEA would cause in the rER. However, the chemical nature of TPGS allows it to reach the rER, where its anti-oxidative properties will protect cells from  $\alpha$ -TEA's oxidative damage. Previous studies have correlated ER stress to  $\alpha$ -TEA's mechanism of action<sup>26</sup>. So it is possible that  $\alpha$ -TEA induces rER stress through ROS that will eventually lead to cell death. In the absence of added GSH,  $\alpha$ -TEA may still provoke oxidative stress in other organelles, predominantly, in the mitochondria. There the damage can compromise the mitochondrial membrane, lead to the release of cytochrome C and initiate the apoptotic pathway. If this pathway is inhibited, the MPTP pathway would still trigger the halt of

glycolysis and the cells would thus perish, if this pathway isn't enough, then elevated levels of ROS may cause enough rER damage to put protein synthesis into a halt and kill the cells.

### **$\alpha$ -TEA's Tumor Specificity in Relation to its Structure and the Production of ROS**

The existing body of evidence suggests that the location of  $\alpha$ -TEA within the cell plays an important role in how it works. But how does it specifically target tumor cells? This concept is not well understood, but it may be explained by the chemical properties of its analogue:  $\alpha$ -Tocopherol. Due to their uncontrolled proliferation, tumor cells will have a high demand for membrane lipids, and so they will receive a higher supply of them through delivery of Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL). Vitamin E can be found in lipoproteins and  $\alpha$ -TEA will likely be located there too. In this way  $\alpha$ -TEA will be administered preferentially to tumor cells and they will accumulate a higher amount of  $\alpha$ -TEA than non-cancerous tissue.

Cancerous cells will be more vulnerable to the production of ROS by  $\alpha$ -TEA than normal somatic cells because they are more likely to have a more delicate ROS homeostasis<sup>13</sup>. In this way, while ROS has the capability of significantly lowering the number of tumor cells, it may have a reduced impact on non-cancerous cells.

The combination of vulnerability to ROS with cellular targeting may better explain the specificity that  $\alpha$ -TEA and other similar compounds may have to tumor cells.

### **$\alpha$ -TEA's Capabilities of Inducing Immunogenic Cell Death**

While there is compelling evidence suggesting that  $\alpha$ -TEA has immunogenic properties in 4T1 cells in vitro<sup>8</sup>, it doesn't appear to involve changes to the direct interaction with lymphocytes via the overexpression of either MHC complex. Furthermore the secretion of

intracellular proteins does not play a role in  $\alpha$ -TEA's method of inducing an immunogenic response after cell death in 4T1 cells. In TUBO cells, the case is very similar, TUBO cells did have a statistically significantly higher expression of calreticulin and Grp 94, however, because the signal level for these markers is so low in this assay, it is questionable whether the spike in the expression of both of these proteins would have any biological significance. Therefore, it appears unlikely that these observations are sufficient to account for the overall impact of  $\alpha$ -TEA on anti-tumor immunity (**Figure 3**). The pathway that is more likely to predominate across different cell lines is one that involves the phosphorylation of TBK-1. The western blot analysis reported in **Figure 3** indicates that  $\alpha$ -TEA caused the phosphorylation of TBK-1 in 4T1 cells. In other experiments (data not shown) we observed pTBK-1 expression in TRAMPC-1 prostate cancer cells, and in a Squamous Cell Carcinoma cell line (SCCVII) that does not produce the STING protein, only when the cells were transfected with the STING gene. The observations strongly suggest that the STING pathway may be activated by  $\alpha$ -TEA. If this is the case, then treatment of tumor cells with  $\alpha$ -TEA may culminate with the secretion of type I interferons and eventually the stimulation of the immune system and tumor suppression.

Characterizing the relationship between the induction of ROS and the effect of  $\alpha$ -TEA on the immunogenicity of tumors will require additional research beyond the scope of this project. Further investigation is required to see whether there is a relationship between ROS and phosphorylation of TBK-1, and whether the presence of pTBK-1 can be attributed to the activation of the STING pathway. It remains a possibility that mitochondrial DNA reaches the cytoplasm after ROS damage causes the mitochondrial membranes to rupture. Subsequently the DNA would be detected by STING, which would initiate the pathway. Yet

there are additional possibilities since STING is also found in the ER, which is an organelle where  $\alpha$ -TEA appears to have a direct impact<sup>27</sup>. ROS produced by  $\alpha$ -TEA in the rER could directly interact with STING, or an oxidized product in the rER could interact with STING after it reacts with ROS.

### **Acknowledgements**

I would like to thank Dr. William Redmond, Dr. Ann Marie Fallon and Dr. Michael McNamara for their advice and guidance. I would also like to thank the staff of the Redmond Lab at the EACRI for their support and for providing assistance during the experiments.

### **References**

1. Kline, K., Lawson, K. A., Yu, W. & Sanders, B. G. Vitamin E and breast cancer prevention: current status and future potential. *J. Mammary Gland Biol. Neoplasia* **8**, 91–102 (2003).
2. Sabharwal, S. S. & Schumacker, P. T. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat. Rev. Cancer* **14**, 709–721 (2014).
3. Constantinou, C., Papas, A. & Constantinou, A. I. Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs: Anticancer Activities of Vitamin E Isomers and Analogs. *Int. J. Cancer* **123**, 739–752 (2008).
4. Klein, E. A. *et al.* Vitamin E and the Risk of Prostate Cancer: The Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* **306**, 1549 (2011).
5. Lawson, K. A. *et al.* Comparison of vitamin E derivatives  $\alpha$ -TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Exp. Biol. Med. Maywood NJ* **229**, 954–963 (2004).
6. Hahn, T. *et al.* Dietary administration of the proapoptotic vitamin E analogue  $\alpha$ -tocopheryloxyacetic acid inhibits metastatic murine breast cancer. *Cancer Res.* **66**, 9374–9378 (2006).

7. Hahn, T., Fried, K., Hurley, L. H. & Akporiaye, E. T. Orally active alpha-tocopheryloxyacetic acid suppresses tumor growth and multiplicity of spontaneous murine breast cancer. *Mol. Cancer Ther.* **8**, 1570–1578 (2009).
8. Hahn, T., Jagadish, B., Mash, E. A., Garrison, K. & Akporiaye, E. T.  $\alpha$ -Tocopheryloxyacetic acid: a novel chemotherapeutic that stimulates the antitumor immune response. *Breast Cancer Res.* **13**, R4 (2011).
9. Li, Y. *et al.* The Vitamin E Analogue -TEA Stimulates Tumor Autophagy and Enhances Antigen Cross-Presentation. *Cancer Res.* **72**, 3535–3545 (2012).
10. Lawson, K. A. *et al.* Novel vitamin E analogue decreases syngeneic mouse mammary tumor burden and reduces lung metastasis. *Mol. Cancer Ther.* **2**, 437–444 (2003).
11. Li, Y. *et al.* Cross-presentation of tumor associated antigens through tumor-derived autophagosomes. *Autophagy* **5**, 576–577 (2009).
12. Simon, H. U., Haj-Yehia, A. & Levi-Schaffer, F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis Int. J. Program. Cell Death* **5**, 415–418 (2000).
13. Cairns, R. A., Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nat. Rev. Cancer* **11**, 85–95 (2011).
14. Youk, H.-J. *et al.* Enhanced anticancer efficacy of alpha-tocopheryl succinate by conjugation with polyethylene glycol. *J. Control. Release Off. J. Control. Release Soc.* **107**, 43–52 (2005).
15. Tesniere, A. *et al.* Molecular characteristics of immunogenic cancer cell death. *Cell Death Differ.* **15**, 3–12 (2008).
16. Kroemer, G., Galluzzi, L., Kepp, O. & Zitvogel, L. Immunogenic Cell Death in Cancer Therapy. *Annu. Rev. Immunol.* **31**, 51–72 (2013).
17. Ishikawa, H., Ma, Z. & Barber, G. N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–792 (2009).
18. Baird, J. R. *et al.* Intratumoral injection of STING ligand promotes abscopal effect. *J. Immunother. Cancer* **2**, P158 (2014).
19. Yan, A., Von Dem Bussche, A., Kane, A. B. & Hurt, R. H. Tocopheryl Polyethylene Glycol Succinate as a Safe, Antioxidant Surfactant for Processing Carbon Nanotubes and Fullerenes. *Carbon* **45**, 2463–2470 (2007).

20. Vadiveloo, P. K., Vairo, G., Hertzog, P., Kola, I. & Hamilton, J. A. Role of type I interferons during macrophage activation by lipopolysaccharide. *Cytokine* **12**, 1639–1646 (2000).
21. Hemmi, H. *et al.* Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* **3**, 196–200 (2002).
22. Buttriss, J. L. & Diplock, A. T. The alpha-tocopherol and phospholipid fatty acid content of rat liver subcellular membranes in vitamin E and selenium deficiency. *Biochim. Biophys. Acta* **963**, 61–69 (1988).
23. Wang, X. & Quinn, P. J. The location and function of vitamin E in membranes (review). *Mol. Membr. Biol.* **17**, 143–156 (2000).
24. Glaumann, H. & Dallner, G. Lipid composition and turnover of rough and smooth microsomal membranes in rat liver. *J. Lipid Res.* **9**, 720–729 (1968).
25. Ault, J. G. & Lawrence, D. A. Glutathione distribution in normal and oxidatively stressed cells. *Exp. Cell Res.* **285**, 9–14 (2003).
26. Tiwary, R., Yu, W., deGraffenried, L. A., Sanders, B. G. & Kline, K. Targeting cholesterol-rich microdomains to circumvent tamoxifen-resistant breast cancer. *Breast Cancer Res. BCR* **13**, R120 (2011).
27. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678 (2008).