UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating Delta Tocopherol as a Novel Dietary Bladder Cancer Chemopreventive Agent

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacology

by

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DEDICATION

To my Family

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PRESENTATIONS

Delta tocopherol inhibits urothelial tumorigenesis in the UPII mutant Ha-ras transgenic mouse model and induces apoptosis via activation of the ATF4/CHOP-DR5 pathway.
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Higher Levels of Secreted S100 A8/9 from Peritumor Perirenal Adipose Tissues are Associated with Renal Cell Carcinoma.
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Zhongbo Liu **Christopher A. Blair**, Xiaolin Zi. AACR 2013 Annual Meeting

ABSTRACT OF THE DISSERTATION

Investigating Delta Tocopherol as a Novel Dietary

Bladder Cancer Chemopreventive Agent

By

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Doctor of Philosophy in Pharmacology

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Professor Xiaolin Zi, Chair

Vitamin E has been the subject of numerous basic and clinical research studies for cancer chemoprevention, which have shown widely varying results. At present, the NIH Office of Dietary Supplements does not consider there to be sufficient evidence for the use of Vitamin E supplementation for cancer prevention. The heterogeneous nature of Vitamin E and the differences in the anti-cancer activities of its components suggests that the study of individual tocopherols may yield more definitive and encouraging results.

Bladder cancer (BCa) presents an ideal opportunity for the evaluation of tocopherols as novel chemopreventive agents. While a majority of newly diagnosed BCa cases are localized and confined to the initiation site on the interior urothelial lining, the high rate of recurrence necessitates long-term follow-up and increases the need for novel agents that can prevent new tumor initiation. As natural products

already present in the diet, with a known safety profile, tocopherols are excellent candidates for application as dietary chemopreventive agents to reduce the recurrence rate of BCa and improve treatment outcomes.

In this study, I demonstrate that delta-tocopherol is more effective than either of the more common alpha- or gamma- tocopherols at inducing apoptosis in human BCa cell lines. I identified the cause of this effect to be induction of endoplasmic reticulum (ER) stress and the unfolded protein response, leading to death receptor 5 mediated apoptosis. Additionally, delta tocopherol treated cells exhibited an autophagy-like phenotype which, when investigated further, provided novel evidence of endoplasmic reticulum specific, ER stress-related autophagy that remains an emerging field of study. Both xenograft and transgenic mouse studies have confirmed the strong anti-tumor and anti-tumorigenesis efficacy of dietary delta tocopherol administration both at the primary tumor initiation site and at distant sites, with no observed negative side effects.

As the first study of delta tocopherol as an individual chemopreventive agent in BCa, these results suggest strong potential for the future development and clinical application of dietary delta tocopherol supplementation to improve the lives and treatment outcomes of BCa patients.

Chapter 1: Introduction

Bladder Cancer Incidence and Current Treatments

There will be an estimated 79,030 new cases of bladder cancer (BCa) and 16,870 related deaths during 2017 (American Cancer Society, 2017). In addition, the estimated prevalence is over 500,000 with a recurrence rate up to 85% (Sievert et. al., 2009). BCa most commonly occurs in older patients, with an average age at diagnosis of 73 years, and over 90% of patients older than age 55 (American Cancer Society, 2017). As with many cancers, the strongest risk factor for BCa is cigarette smoking, though other major risk factors include age, chemical exposure, and chronic bladder problems such as bladder stones and infections. Whites are roughly twice as likely as African Americans to be diagnosed with BCa, though black patients have lower overall survival rates. Men are roughly four times as likely as women to be diagnosed with BCa, though women have lower 5-year survival rates (79% vs 74% in white men vs. white women, 69% vs. 54% in black men vs. black women) (American Cancer Society, 2017). Because of the high frequency of tumor recurrence, the lifetime need for surveillance, difficult treatment of recurrent tumors, and the high cost of complications associated with treatments, BCa is a major public health burden in the United States (US). Approximately $3.7 billion is spent in the US each year on BCa treatment (Mostafid et. al., 2015). More specifically non-muscle invasive bladder cancer (NMIBC), in which cancerous cells are confined to the urothelial lining and have not yet invaded the smooth muscle of the bladder wall, is the most common neoplasm of the urinary tract, accounting for over 80% of newly diagnosed bladder cancer cases (Aldousari and

Kassouf, 2010).

Despite current treatments such as transurethral resection, and intravesicular BCG or mitomycin C, BCa is associated with high risk of recurrence and/or progression to invasive and metastatic disease (American Cancer Society, 2017). The standard of care treatment for NMIBC, Transurethral Resection of Bladder Tumor (TURBT), involves the insertion of a cystoscope through the urethra and direct removal of localized tumors by the surgeon with a tool. While highly effective, TURBT is often paired with targeted radiation therapy or intravesical BCG (a mycobacterial immunotherapy) or intravesical chemotherapy treatment (in which the therapeutic agent is directly delivered into the bladder using a catheter) shortly after surgery to minimize the chance of any stray cancer cells initiating a new tumor. TURBT patients require continuous surveillance via cystoscopy for the rest of their lives, as even completely successful TURBT procedures still carry a high risk of recurrence months or years after surgical intervention. (Cao et al. 2015) This need for life-long invasive surveillance and treatment places NMIBC among the most expensive cancers to treat on a per-patient basis and it represents a major public health challenge (Seivert et. al., 2009; Mostafid et. al., 2015). The development of a safe, low cost and oral drug for preventing and treating recurrence and progression of NMIBC is a clear priority in urologic oncology and could dramatically reduce the cost of long-term BCa treatment and improve patients' quality of life. The need for novel BCa chemopreventive agents has been further exacerbated in recent years due to shortages of BCG, which is provided by only a single supplier and which as an organism, rather than a molecule, is highly

vulnerable to delays in production due to contamination which can shut down the entire production process, which already takes up to three months per batch under normal circumstances (Messing, 2017).

Patients diagnosed with more advanced bladder cancer may also benefit from the development of anti-tumor agents due to the relatively low number of treatments available for metastatic BCa. Patients with muscle-invasive BCa (MIBC), which has penetrated past the urothelial lining and into the smooth muscle of the bladder wall, undergo partial or radical cystectomy to remove part or all of the bladder. Intravesical therapy is also ineffective in MIBC patients as the therapeutic agents can no longer reach the entire tumor, necessitating the use of systemic chemotherapy with significantly worse side effects. (Chou et al. 2016) Development of additional agents with minimal off-target toxicity could open the door to new combinations of drugs or reductions in the necessary dosages of existing chemotherapeutics and an improvement in treatment outcomes and patients' quality of life.

Vitamin E as a chemopreventive agent

Vitamin E is a fat-soluble group of natural compounds occurring in dietary sources known primarily for its antioxidant activity (Traber, 2009). Natural vitamin E consists of eight compounds, the four more prevalent tocopherols (alpha- (α), beta- (β), gamma- (γ), and delta- (δ)) and the four much less prevalent corresponding tocotrienols (Muller et. al., 2010). The most abundant tocopherols in dietary sources are the α and γ forms, which are found in common foodstuffs such as peanut, corn, and

soybean oils. δ tocopherol (δ-T) is abundant in fewer sources, most notably edamame and raspberries. (United States Department of Agriculture 2018) Tocotrienols are less common in dietary sources, mostly occurring in seeds and plant oils ((Theriault et. al. 1999)), which may be due to the conversion of tocotrienols into tocopherols within plants as intermediates in the synthesis of α tocopherol (α-T) (Pennock, 1983). Synthesis of tocopherols is notably more successful than synthesis of tocotrienols, which have only been produced with comparatively poor purity and yields (Netscher, 2007), increasing the attractiveness of tocopherols as potential therapeutically useful agents compared to tocotrienols, particularly in the case of the less abundant isoforms which would be inefficient to seek out in the diet. To reach potentially therapeutic concentrations of tocotrienols in the bloodstream, prohibitively large amounts of dietary tocotrienol sources would have to be consumed, requiring an adult human to eat between 1.5-4 kg of grain sources such as barley, oats, or wheatgerm (Sen et. al., 2007). The bioavailability and half-life of tocotrienols being significantly worse than those of the tocopherols (Yap et al., 2003, Leonard et. al., 2005) combined with the more efficient synthesis of tocopherols than tocotrienols indicates that a focus on tocopherols may be most likely to lead to the development of a clinically relevant agent.

Studies of dietary Vitamin E intake and supplementation as chemopreventive agents have demonstrated mixed results, casting doubt on its use either as an individual agent or as a supplement to other treatment methods (Cook-Mills et al., 2013; Greenberg, 2005; Lee et al., 2005; Lonn et al., 2005). Calling these studies clinical

trials on Vitamin E, however, is misleading and may unfairly cast doubt on Vitamin E's therapeutic potential due to their heavy focus on α-T.

Lung cancer cohort studies have found an inverse relationship between vitamin E consumption and risk of lung cancer, and showed protective effects of vitamin E intake in current smokers (Smith-Warner et. al., 2003). Additionally, these studies found that levels of α-T in the serum of lung cancer patients were lower than levels found in matched control patients (Ju et. al., 2010). An additional study found that tocopherol intake reduced lung cancer risk by 34-53%, though the increased intake of α-T in this study was accompanied by proportionally increased γ-T intake, so the exact components of the vitamin E mixture which caused this risk reduction remains unclear (Mahabir et. al., 2008). Other studies of vitamin E and cancer risk have shown much less encouraging results. Most notably, the large scale SELECT (Selenium and Vitamin E Cancer Prevention Trial) study failed to show any protective effect of Vitamin E supplementation against prostate or any other cancers over the 5.5 year average supplementation time (Lippman et. al., 2009), and in fact the follow up on study participants found an increased overall risk of prostate cancer in those patients who received Vitamin E supplementation (Klein et. al., 2011). An additional notable observation in the SELECT study was a reduction in γ-T plasma levels in patients supplemented with α-T (Lippman et. al., 2009). Trials of Vitamin E for cancer and cardiovascular disease prevention in women also found no overall benefit of Vitamin E supplementation for either indication (Lee et. al., 2005; Wu et. al., 2014). Long duration Vitamin E supplementation for 10 or more years has also been linked to reduced

bladder cancer mortality, though regular use for a shorter duration was not (Jacobs et. al., 2002).

An important flaw in the study of dietary Vitamin E supplementation that may be responsible for the disappointing results of large clinical studies is the fact that the primary form of Vitamin E in the American diet is γ-T due to its prevalence in many of the commonly used dietary oils (Dietrich et. al., 2006; Jiang et. al., 2001), however most studies of Vitamin E use alpha-tocopherol acetate or mixed Vitamin E consisting of mostly α-T (Miller et. al., 2005). Since α-T supplementation was shown to reduce the availability of γ and δ Tocopherols in serum (Brigelius-Flohe and Traber 1999, Traber 2007), this may be responsible for the observed increase in prostate cancer risk in the SELECT study. The impact of excessive α-T supplementation in these studies likely blocks any biological activity of other tocopherol isoforms that patients may have consumed in their diets, rendering the studies useful only as evaluations of alpha-tocopherol specifically rather than heterogeneous Vitamin E or its other components.

Studies of individual Vitamin E components

The rationale for using α-T as the Vitamin E component in clinical trials was in and of itself not necessarily flawed. After entering the bloodstream, dietary Vitamin E reaches the liver, where α-T is strongly bound by alpha-tocopherol transport protein (αTTP) and prepared for delivery to the rest of the body (Hosomi et. al., 1997, Sontag and Parker 2002). αTTP has significantly lower binding affinity for other tocopherol isoforms and tocotrienols (Brigelius-Flohe and Traber 1999, Traber 2007), which

results in unbound tocopherols being metabolized by cytochrome P450 and ω-hydroxylase into carboxychromanols, hydroxycarboxychromanols, carboxyethyl-hydroxychromanols, and their derivatives, which are excreted in the urine. (Jiang 2014, Birringer et. al., 2002, Lodge et. al. 2001). These characteristics of α-T absorption have led to its wide consideration as the "primary" dietary tocopherol and combined with its ease of synthesis to lead to its use in clinical supplementation studies in which the consistency of supplement intake was of critical importance.

The impact of α-T on other tocopherol isoforms in the human body necessitates the study of the other primary forms of tocopherol (γ and δ) as individual chemopreventive agents, in order to remove the potential confounding effect of α-T's impact on the bioavailability of these other forms. γ-T, more prevalent in diet in the United States (Jiang et. al., 2001), has been the subject of numerous tumorigenesis studies by C.S. Yang et al., who found protective effects of dietary γ-T supplementation in lung, colon, mammary, and prostate tumorigenesis (Lu et. al., 2010; Li et. al., 2011; Lambert et. al., 2009; Ju et. al., 2009; Lee et. al., 2009). δ-T, however, has been minimally studied as an individual agent. One study indicated that δ-T is more effective than both α-T and γ-T in lung tumorigenesis inhibition and resulted in high accumulation of tocopherol side-chain metabolites in the colon and bladder (Li et. al., 2011). Recent studies have also demonstrated that δ-T was more active than γ-T in inhibiting AOM-induced colon aberrant crypt foci in rats as well as the growth of many cancer cell lines in culture and lung cancer H1299 cells in a xenograft tumor model, whereas α-T was ineffective (Lambert et. al., 2009; Ju et. al., 2009). The inhibitory actions were mostly associated

with quenching of reactive oxygen and nitrogen species, lowered prostaglandin E2 levels, anti-inflammatory activity, enhanced apoptosis as well as decreased phosphorylated AKT levels and increased levels of PPAR-γ, p21 and p27 (Lu et. al., 2010; Li et. al., 2011; Lambert et. al., 2009; Ju et. al., 2009; Lee et. al., 2009). Additionally, dietary administration of γ- and δ-, but not α-Ts have demonstrated tumorigenesis inhibition in Estrogen Receptor positive breast cancer mouse models (Smolarek and Suh, 2011). As our preliminary data show that δ-T is more effective than both α- and γ-T's against multiple human bladder cancer cell lines, the further study of δ-T supplementation as an individual chemopreventive agent is necessary.

The Unfolded Protein Response and Endoplasmic Reticulum Stress

An emerging field of study in cancer chemotherapeutic development is the Unfolded Protein Response (UPR) and endoplasmic reticulum (ER) stress. The UPR is a versatile response initiated when a cell detects the dangerous accumulation of unfolded or misfolded proteins in the ER, which has both pro-survival and pro-apoptotic potential outcomes. (Lu et. al., 2014) The ER is a critical organelle in the cell that serves, among its numerous functions, as the site for much of the protein synthesis, folding, and processing necessary for cell growth and survival (Sano and Reed, 2013). In situations of increased protein synthesis it is possible for an accumulation of unfolded proteins to begin within the ER, which stresses the system and disrupts ER homeostasis (Bravo et. al., 2013). Tumor cells generally operate with a higher level of background ER stress caused by the increased protein load on the ER caused by their heightened rate of

mitosis and the poor physical conditions of hypoxia and poor vascularization in the tumor environment (Travers et. al., 2000; Tsai et. al., 2010) The ER membrane has sensors embedded which detect the presence of increasing amounts of unfolded proteins and initiate the UPR, which attempts to increase the rate of protein folding by promoting the expression of additional folding-chaperone proteins and decreasing the rate of other protein synthesis (Luo and Lee, 2013). Three major ER stress sensing molecules, PERK, IRE1α, and ATF6, each regulate a branch of the UPR, with PERK and IRE1α playing an important role in chronic ER stress response (Lu et. al., 2014). Both PERK and IRE1α detect accumulation of unfolded proteins when folding chaperone BiP, also known as Grp78, which under normal conditions remains bound to either sensor, binds to its unfolded protein substrate and releases from either PERK or IRE1α, allowing them to oligomerize (Bertolotti et. al., 2000, Carrara et. al., 2015).

PERK, a type I transmembrane kinase embedded in the ER, detects accumulation of unfolded proteins and responds by oligomerizing and trans-autophosphorylating, leading to phosphorylation of eukaryotic translation initiation factor-2 (eIF2α) and reduction in the translation of most mRNA transcripts (Walter and Ron, 2011). Transcription factor ATF4, however, is selectively upregulated by this process, and functions to increase ER protein folding capacity in the short term and induces macroautophagy. However, during extended periods of ER stress ATF4 induces CHOP expression, which in turn activates pro-apoptotic signaling via Death Receptor 5 and the BCL-2 family genes (Harding et. al., 2003)

IRE1α, and ER transmembrane protein with both a kinase and endoribonuclease

domain, induces a highly conserved branch of the UPR when it detects unfolded protein accumulation. IRE1α dimerizes and trans-autophosphorylates to activate its endoribonuclease domain, which in turn splices XBP1 mRNA into XBP1s mRNA, yielding a unique transcription factor (Shen et. al., 2001, Yoshida et. al., 2001, Calfon et. al., 2002). XBP1s, which can heterodimerize with other transcription factors in different tissues (Hetz, 2012), primarily functions to control expression of genes related to maintaining ER protein homeostasis (Lee et. al., 2003, Acosta-Alvear et. al., 2007). The endoribonuclease domain of IRE1α also acts directly on multiple other RNAs including mRNAs, microRNAs, and rRNAs in a sequence and structure-specific manner, a process known as regulated IRE1-dependent decay (RIDD) (Hollien and Weissman, 2006; Maurel et. al., 2014). RIDD also contributes to the survival of cells in the UPR by degrading CHOP mRNA and preventing the accumulation and activation of this pro-apoptotic transcription factor (Lu et. al., 2014).

If the UPR is unable to resolve the accumulation of unfolded proteins, the pro-survival activity of the UPR will attenuate and the stressed cell will be committed to apoptosis (Shore et. al., 2011) mediated by both the intrinsic and extrinsic apoptosis pathways, regulated by the Bcl-2 protein family and Death Receptor 5 (DR5), respectively (Cory and Adams, 2002, Yamaguchi and Wang, 2004). Continued PERK signaling's suppression of protein translation prevents long-term cell survival and selectively enhances CHOP expression, leading to DR5 expression, suppressing anti-apoptotic Bcl-2 expression, and increasing expression of pro-apoptotic Bcl-2 gene family members (Urra et. al., 2013). Persistent IRE1α activation similarly results in

increasing RIDD activity and degrades the continued ability of the ER's protein folding machinery to alleviate unfolded protein accumulation (Hollien and Weissman, 2006; Han et. al., 2009). Ultimately, the signaling of both intrinsic and extrinsic apoptotic pathways converge on the activation of the effector caspase 3 and subsequent cell death.

DR5 is a receptor for TRAIL (TNF-related apoptosis inducing ligand), an apoptosis inducing cytokine, which has been shown to selectively kill cancer cells through activation of DR5. (Graves et. al., 2014) DR5 upregulation has been demonstrated to overcome the effect of loss of function in the p53 tumor suppressor gene to induce apoptosis in colon cancer models (Thamkachy et. al., 2016). Application of a DR5 agonist antibody AMG665 has also been demonstrated to combine synergistically with TRAIL to promote cancer cell death both *in vitro* and *in vivo* (Graves et. al., 2014), indicating therapeutic potential for DR5 expression increasing agents. Anti-leukemic agents, including doxorubicin, etoposide, and Ara-C have also been demonstrated to increase DR5 expression and yield improved results when combined with TRAIL treatment (Wen et. al., 2000). While DR5 mRNA is transiently decayed by IRE1α's RIDD activity, the persistent upregulation of DR5 mRNA expression by the PERK/ATF4/CHOP signaling pathway eventually leads to accumulation of DR5 protein and ligand-independent DR5 activation in cases of chronic ER stress and activation of caspase-8 mediated apoptosis (Lu et. al., 2014).

While the importance of apoptosis as a mechanism of programmed cell death induced by many cancer therapies is well documented, other mechanisms of

programmed cell death remain less well-defined and require further study. Autophagy, or self-eating, is a process by which a cell responds to stress sources such as starvation or hypoxia by inducing vacuolization of cytoplasmic contents and their subsequent degradation via the fusion of lysosomes with the resulting autophagosomes (Kroemer et al. 2008). This process is characterized by the following steps, in order: formation of a phagophore membrane, conjugation of autophagy-related (Atg) proteins, aggregation and insertion of LC3 protein into the membrane of the phagophore, vacuolization of targets by the phagophore, and fusion of the newly formed autophagosome with lysosomes to form an autolysosome (Glick et al. 2010). Autophagy's role in cell death remains to be completely clarified, as whether the outcome of autophagy is ultimately pro- or anti- survival is heavily context dependent, with different cell types and healthy vs. cancerous cells responding differently to autophagy inhibition in experimental settings (Hara et al. 2006; Komatsu et al. 2006; Nelson and Baehrecke 2014). While autophagy is often blamed for the process of Autophagic Cell Death, an apparently apoptosis- independent mechanism of programmed cell death characterized by a strong autophagic phenotype (Shintani and Klionsky 2004; Tsujimoto and Shimizu 2005; Codogno and Meijer 2005), there remains some doubt as to whether Autophagic Cell Death truly consists of cell death caused by autophagy, or if it simply cell death with autophagy as a contributing factor (Kroemer and Levine, 2008). There is evidence of autophagy playing a role as an upstream effector of apoptotic cell death, as autophagy inhibition by Atg knockdown has been shown to suppress apoptosis in HIV induced CD4+ T cells, photoreceptors subjected to oxidative stressors, and

osteosarcoma cells (Espert et al. 2006; Kunchithapautham and Rohrer 2007; Crighton et al. 2006).

Notably, ER stress inducing agents have been linked to autophagy induction in addition to their role in causing apoptotic cell death. ER stress inducers have been shown in colon and prostate cancer cell lines to induce autophagy as a compensatory mechanism linked to the UPR to alleviate stress (Ding et al. 2006). Expansion of the ER in response to ER stress has been identified in both mammalian and yeast cells, featuring enlargement of both the ER surface area and volume as a mechanism to increase dedicated protein folding space and disperse the load on folding chaperone proteins (Bernales et al. 2006). To date, the evidence for selective autophagy has been limited, with the strongest findings coming in yeast cells, where selective degradation of ER membrane, referred to as "ER-phagy" or reticulophagy, has been identified via a mechanism independent of the canonical autophagic machinery (Schuck et al. 2014). Additionally, ER stress can induce the formation of ER whorls, rounded ER structures that can be similarly selectively degraded independently of normal autophagic mechanisms (Schuck et al. 2014). While these findings suggest that ER-specific degradation occurs as a method of restoring or preserving homeostasis in response to ER stress, there has to date been little evidence of this phenomenon's occurrence in mammalian cells.

Targeting the UPR for Cancer Therapy

The pharmacological targeting of UPR activity has been identified as a novel

strategy for drug development by numerous companies, resulting in one FDA-approved drug (Bortezomib) and numerous ongoing trials. Drugs such as Bortezomib (Adams and Kauffmann, 2004), Carfilzomib (Vij, 2012), Nelfinavir (Gills et. al., 2007), and Marizonib (Chauhan et. al., 2006) all target the proteasome, which results in accumulation of ubiquitinated proteins that are no longer cleared effectively (Crawford et. al. 2011) and induces ER stress and subsequent apoptosis. Direct targeting of the ER stress response is also being evaluated in clinical trials of inhibitors of protein folding chaperone BiP such as DHA (Begum et. al., 2014) and PAT-SM6 (Hensel et. al., 2013). Inhibition of protein folding by these agents is intended to induce UPR-mediated cell death by removing the cancer cells' ability to successfully resolve the pro-survival side of the UPR and force a switch to apoptosis. Inhibitors of ER stress sensors PERK (6-shogaol (Hu et. al., 2012), GSK2656157 (Axten et. al., 2013), GSK2606414 (Axten et. al., 2012)) and IRE1α (STF-083010 (Papandreou, 2011), MKC-3949 (Mimura et. al., 2012)) are also undergoing preclinical evaluation. The development of these novel small molecule inhibitors of certain portions the UPR's activity provides an opportunity to identify additional agents with mechanisms of action that may work synergistically with new drugs to enhance their efficacy or lower the necessary therapeutic dose in order to reduce potential side effects. Novel agents which can induce UPR activation and ER stress could potentially be combined with inhibitors of specific pro-survival UPR components or proteasomal inhibitors to achieve synergistic improvements to the cytotoxicity of cancer drugs.

Rationale for Current Studies

The lack of a convincing consensus on the efficacy of Vitamin E supplementation for cancer prevention demands more specific study of the isoforms that make up dietary Vitamin E. As a class of molecules already common in the diet and therefore having favorable safety characteristics, the tocopherols present attractive candidates for development as novel chemopreventive agents with relatively low barriers to their clinical usefulness. However, existing studies on the individual components of heterogeneous Vitamin E demonstrated that the α-T previously used in clinical trials may have been doomed to failure from the start, as in many cases α-T demonstrated the weakest effect of the tocopherols in many key aspects of cancer drug development. While the dietarily widely available γ-T has been the subject of some studies, as have the tocotrienols, they still have not overcome the mixed perception of Vitamin E caused by the poor results of α-T focused studies. Our preliminary data has demonstrated that δ-T was more effective than both α- and γ-T in BCa. The effect of δ-T on BCa, and indeed on most other cancers, has not been reported. There is also an absence of study on the potential interaction between δ-T and the ER stress response and UPR induction, and study of this link may provide the rationale for novel prevention and combination therapy trials that could significantly improve the effect of many novel UPR targeting small molecule drugs. Bladder cancer is an extremely attractive target for the development of novel chemopreventive agents as a result of the combination of its recurrence characteristics, organ site, and the burdens placed on its patients. The

bladder presents an excellent environment for the activity of compounds that are excreted in urine, as δ-T and its metabolites are (Hensley et. al., 2004). The prevalence of tumorigenesis initiation along the interior urothelial lining of the bladder in BCa recurrence makes δ-T an exciting potential agent for development as a novel and efficacious agent for BCa chemoprevention through dietary supplementation, while also allowing the potential for development as an intravesical agent if such an approach would help avoid pharmacokinetic issues that may be caused by dietary interference from α-tocopherol intake. The UPR is also a promising target for the development of novel therapeutic agents and approaches, as the critical nature of this mechanism renders it well conserved in different tissue sites and increases the chances that an agent developed in any specific tumor type will have broader applicability in other cancers.

Chapter 2: Evaluation of α, γ, and δ Tocopherols as Potential Chemopreventive Agents

Introduction

Clinical studies of Vitamin E supplementation in cancer patients have demonstrated mixed results. While some studies demonstrated a modest reduction in cancer incidence with Vitamin E supplementation, others have shown no benefit or even slightly elevated cancer incidence (Smith-Warner et. al., 2003; Ju et. al., 2010; Mahabir et. al., 2008; Lippman et. al., 2009; Klein et. al., 2011; Lee et. al., 2005; Wu et. al., 2014). Chemical structures of tocopherols differ in the number and position of methyl groups on the chromanol ring. α-T is 5-, 7- and 8-trimethylated, whereas γ-T is 7, 8-dimethylated and δ-T is 8-methylated. All the tocopherols are antioxidants; however, γ-T and δ -T are more effective than α-T in trapping reactive nitrogen species, due to the unmethylated carbon at the 5-position of the chromanol ring (Muller et. al., 2010). (**Figure 2.1)** Despite these differences, clinical studies of "Vitamin E" have relied on supra-nutritional supplementation of α-T (Miller et. al., 2005) rather than mixtures of tocopherols, which would produce significant variation between studies even if it may have more accurately reflected dietary tocopherol intake. The usefulness of these results therefore depends heavily on the activity of just one component of a heterogeneous class of molecules. This aspect of the large-scale clinical trials that have been performed to date has prematurely cast doubt over the effectiveness of Vitamin E components as chemopreventive agents by ignoring the diversity of Vitamin E isoforms in the diet and available synthetically. There is increasing evidence that the

methodology of these clinical trials may also have been responsible for some of the inconsistencies in their results. Particularly, the pharmacokinetics of dietary tocopherols significantly favor the prevalence of α-T in the bloodstream (Hosomi et. al., 1997, Sontag and Parker 2002). α-T is protected from degradation in the liver by αTTP, which has a significantly higher affinity for α-T than other tocopherol isoforms and tocotrienols (Brigelius-Flohe and Traber 1999, Traber 2007). While α-T is packaged for distribution to the rest of the body through the bloodstream, the other tocopherols undergo metabolism in the liver into other chromanol containing compounds, which are then excreted in the urine (Jiang 2014, Birringer et. al., 2002, Lodge et. al. 2001).

Materials and Methods

Cell Culture: Cell lines were original acquired from ATCC and stored in liquid nitrogen, until thawed and maintained in recommended growth media (RT4, T24-McCoy's 5A, UMUC3-EMEM, UroTsa-Low glucose DMEM) supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, at 37° C and 5% CO_2 with normal atmospheric oxygen levels.

Compounds: Tocopherol stock solution was generously provided by Prof. C.S. Yang (Rutgers University) at a concentration of 50 mM. Tocopherols were prepared for use in cell culture by further solubilizing in absolute ethanol to overcome poor solubility issues before diluting into large volumes of the appropriate complete cell culture media to produce a stock solution for serial dilution. Fresh tocopherol solution was prepared

for each experiment, as separation occurs with diluted tocopherols in cell culture media during storage.

MTT Assay: Cells were plated at $2x10^4$ cells/well in 24 well tissue-culture plates and allowed to anchor overnight before growth media was replaced with 500 uL of tocopherol-supplemented or vehicle control medium. Plates were then incubated for 72 hours before addition of 150 uL of MTT dye solution (3mg/mL in 1x PBS) to each well, and incubated again for 1.5 hours. The media+MTT solution was then removed and the purple formazan crystals remaining in the wells were dissolved in acidified isopropanol (4% 1N HCl) for absorbance measurement at 570nm. Notably, reaction between MTT dye and the tocopherol-containing media was only detectable at concentrations well in excess of those used in this study.

Flow Cytometry: RT4 cells were plated at 100,000 cells/well in 6 well plates and allowed to anchor overnight. Cells were then treated with tocopherols at desired concentrations for 24 hours, trypsinized, and incubated with GUAVA ViaCount Reagent for 10 minutes, after which cell viability was analyzed by flow cytometry.

Results

RT4 NMIBC cells treated with α, γ, and δ tocopherols for 72 hours and analyzed using an MTT metabolic assay for cell viability exhibited IC50s of 49.95μM, 36.75 μM, and 5.63 μM, respectively. UM-UC-3 MIBC cells exhibited IC50s of 21.07 μM for γ-T and 9.59 μM for δ-T, and T24 MIBC cells exhibited IC50s of 8.98 μM for γ-T and 7.33 μM for δ-T. In both MIBC cell lines, sufficient concentrations of α-T to reach 50% cell viability were not attainable in cell culture media. **(Figure 2.2)**

Flow cytometry was next performed on RT4 cells using α, γ, and δ tocopherols each administered at 5 μM concentration for 24 hours, as a direct measure of cell viability rather than the metabolic activity reported by the MTT assay. In this assay, only δ-T induced a notable decrease in cell viability, a 21.13% reduction (73.4% viability δ-T treated vs 96.53% viability vehicle control, $p<.0001$). **(Figure 2.3)**

To ensure that the use of δ-T as a chemopreventive agent would be safe for healthy cells a non-cancerous urothelial cell line immortalized using expression of the SV40 large T antigen (Rossi et al. 2001), UroTsa, was treated in an MTT assay as previously described for the BCa cell lines. An IC50 value of 25.46μM was obtained from this experiment. **(Figure 2.4)**

Due to the primary perceived dietary role of tocopherols and Vitamin E as an antioxidant, an ROS assay was performed using RT4 and UM-UC-3 cells to determine if the antioxidant activity of δ-T played a role in its cytotoxic effect, however no difference was identified between treated and untreated cells (data not shown). Additionally, the

primary urinary metabolite of δ-T, Delta CEHC, was tested for cytotoxic effects against RT4 NMIBC cells, but no effect was observed. **(Figure 2.5)**

Discussion

Mixed outcomes of Vitamin E supplementation clinical trials have necessitated the analysis of dietary Vitamin E's constituent compounds to obtain a more accurate understanding of it's potential as a chemopreventive agent (Cook-Mills et al., 2013; Greenberg, 2005; Lee, Cook, Gaziano, et al., 2005; Lonn et al., 2005). In order to determine which of the tocopherols to follow with further mechanistic study, I first examined the growth inhibitory effects of δ, γ & α-Ts in NMIBC cell line RT4, and muscle invasive BCa cell lines T24 and UMUC3. Choosing cell lines representative of both NMIBC and MIBC allows validation of the selected tocopherols both as an agent for the prevention of tumor initiation after successful surgical treatment of early-stage BCa and as a potential intervention in patients with more advanced disease. Furthermore, *in vitro* comparison of the tocopherols allows the analysis of each isoform's efficacy in the complete absence of dietary tocopherols that could otherwise alter the measurable outcomes in clinical or *in vivo* studies.

In MTT metabolic activity assays of all three tested cell lines, we found that α-T failed to demonstrate significant cytotoxic effects, further supporting the need to assess the other tocopherols individually. γ-T, for which there is evidence of anti-cancer efficacy in literature (Lu et. al., 2010; Li et. al., 2011; Lambert et. al., 2009; Ju et. al., 2009; Lee et. al., 2009), killed all three cell lines more efficiently than α-T, though its

effectiveness against NMIBC cell line RT4 was disappointing. Of the tested tocopherols, δ-T showed the most promise as a potential novel drug candidate due to its strong efficacy against all three BCa cell lines. While δ-T is less prevalent dietarily than α-T and γ-T , and suffers from pharmacokinetic interference from dietary α-T, there are still available dietary sources rich in δ-T (United States Department of Agriculture, 2018), and supra-nutritional δ-T supplementation may also prove to be more effective than traditional Vitamin E supplementation methods.

All three tocopherols were also evaluated after 24 hours of 5uM treatment in RT4 NMIBC cells by flow cytometry, which further supported the choice of δ-T for further study. As they represent the most desirable form of BCa to target pharmacologically for chemopreventive purposes, efficacy against NMIBC cells is essential for development of a new dietary or oral supplementation agent. Of the tested tocopherols, only δ-T induced a significant reduction in RT4 cell viability (73.4% cell viability at 24 hours vs 96.53% viability for vehicle control treated cells.) As a result, I chose δ-T as the compound to focus on in pursuit of further pre-clinical mechanistic studies in order to maximize the potential for a clinically applicable outcome.

To further confirm that δ-T has the potential for development as a therapeutic agent from a safety perspective, I tested the cytotoxicity of δ-T on UROTsa cells, an immortalized bladder epithelial cell line commonly used as a healthy cell control for bladder cancer studies (Rossi et al. 2001). Reassuringly, the IC50 of δ-T treatment on UROTsa cells was roughly 25μM, significantly higher than the IC50s for all tested cancerous cell lines. In combination with the known safety of oral Vitamin E

consumption (National Institutes of Health, 2018), these results present a strong rationale for the further development of dietary or orally supplemented δ-T as a novel BCa chemopreventive agent.

RT4 cells were also treated with the most prevalent urinary metabolite of δ-T, the hydrophilic δ-CEHC, however no cytotoxic effect was observed with this compound. As tocopherols primarily enter cells by lipophilic interaction with the cell membrane (Wang and Quinn, 2000), it is likely that δ-CEHC simply is unable to enter the cell in order to exert any cytotoxic effect.

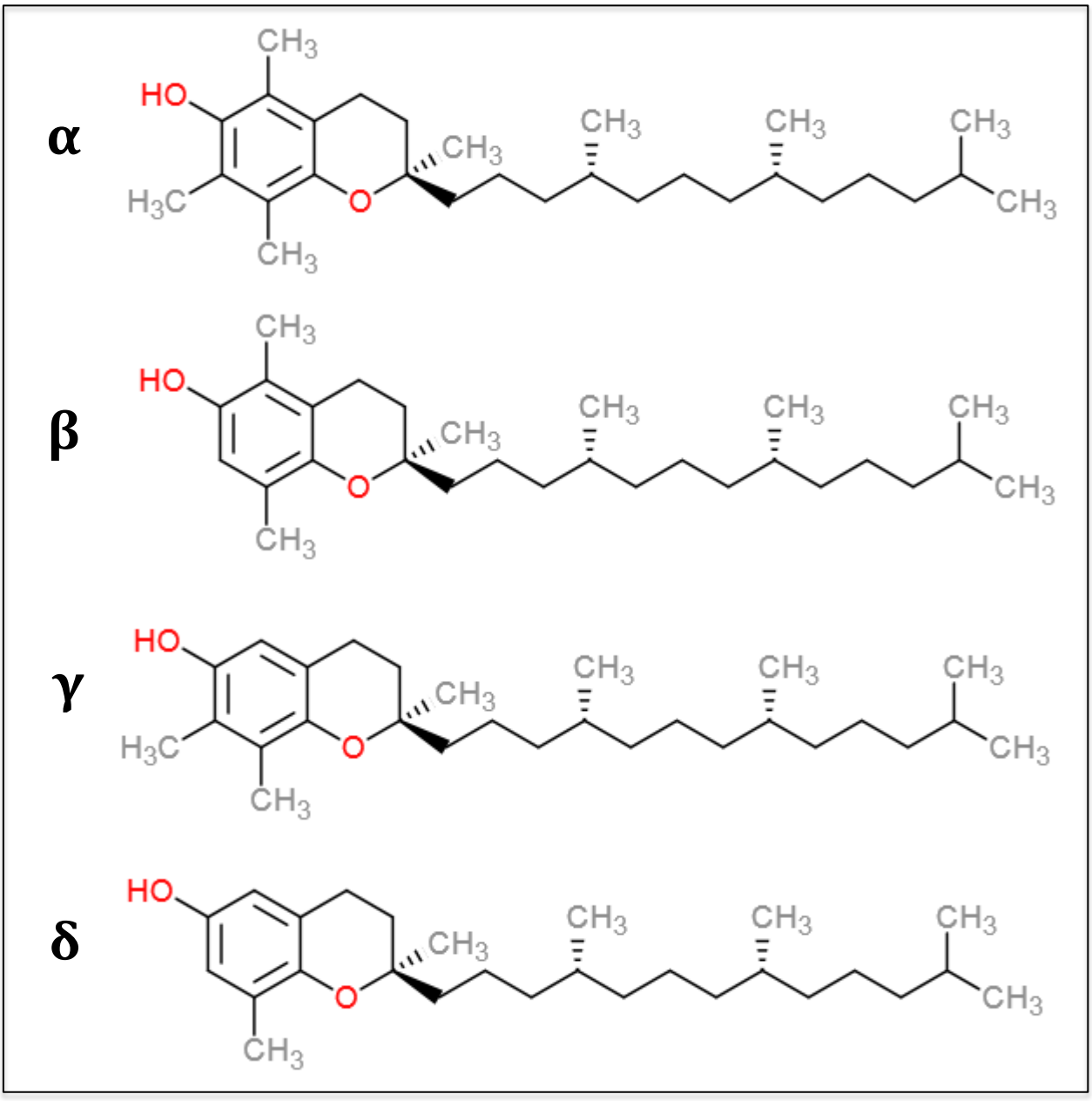

Figure 2.1 Chemical structures of the tocopherol components of Vitamin E

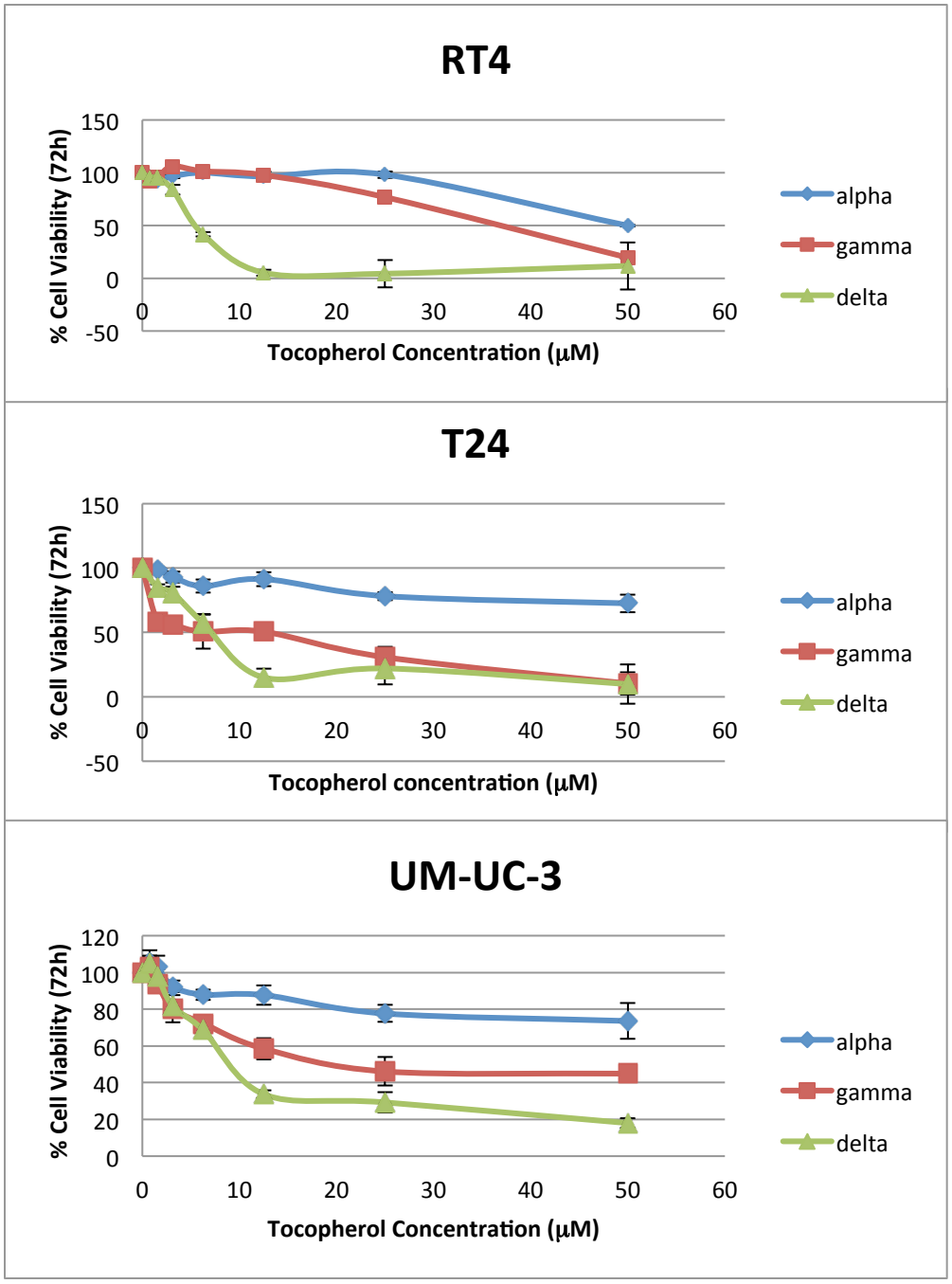

Figure 2.2 MTT Assay comparing tocopherol efficacies against BCa cell lines

Cells treated with α, γ, and δ tocopherols for 72 hours. RT4 IC50s of α: 49.95μM, γ: 36.75μM, δ: 5.63μM. UM-UC-3 IC50s γ: 21.07μM, δ: 9.59μM. T24 IC50s γ: 8.98μM δ: 7.33μM

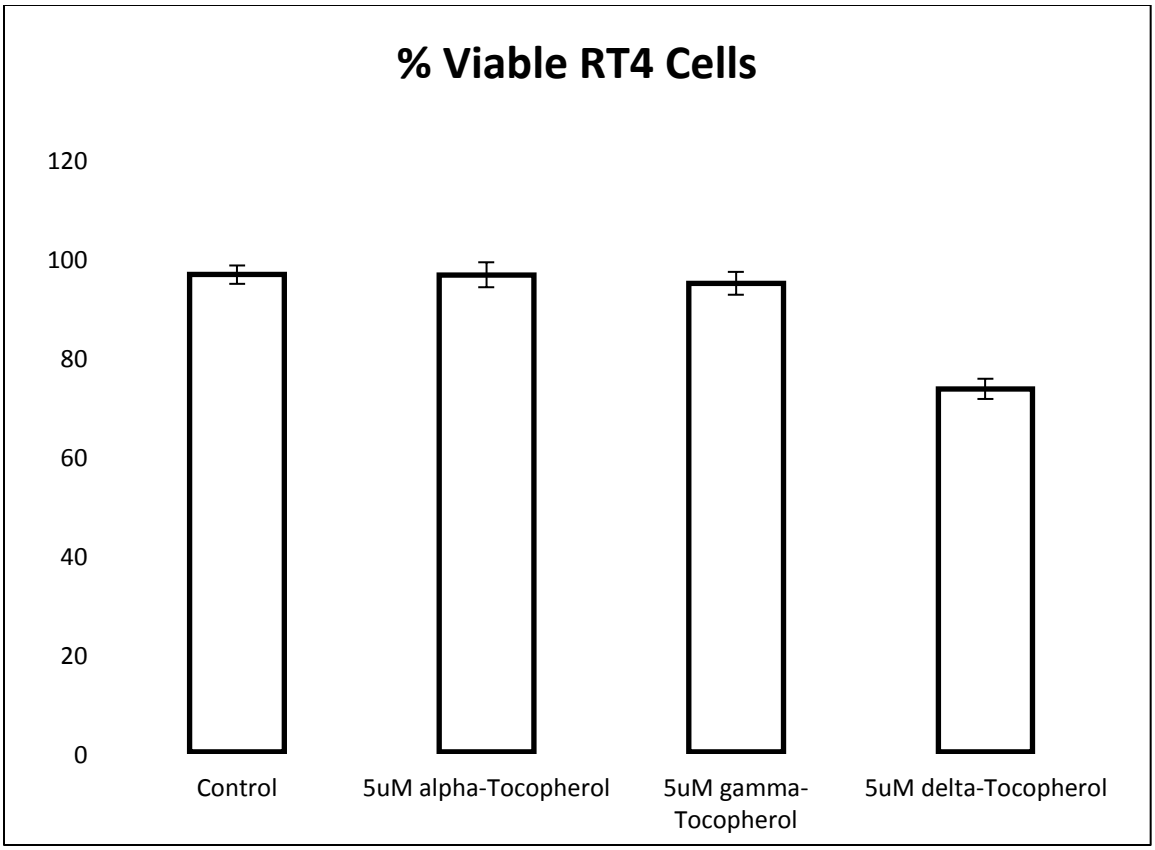

Figure 2.3 Direct assessment of the effects of tocopherols on cell viability by flow cytometry

After 24 hours of treatment with all three tested tocopherols, only δ-T induced a notable decrease in cell viability, a 21.13% reduction (73.4% viability δ-T treated vs. 96.53% viability vehicle control, $p<.0001$).

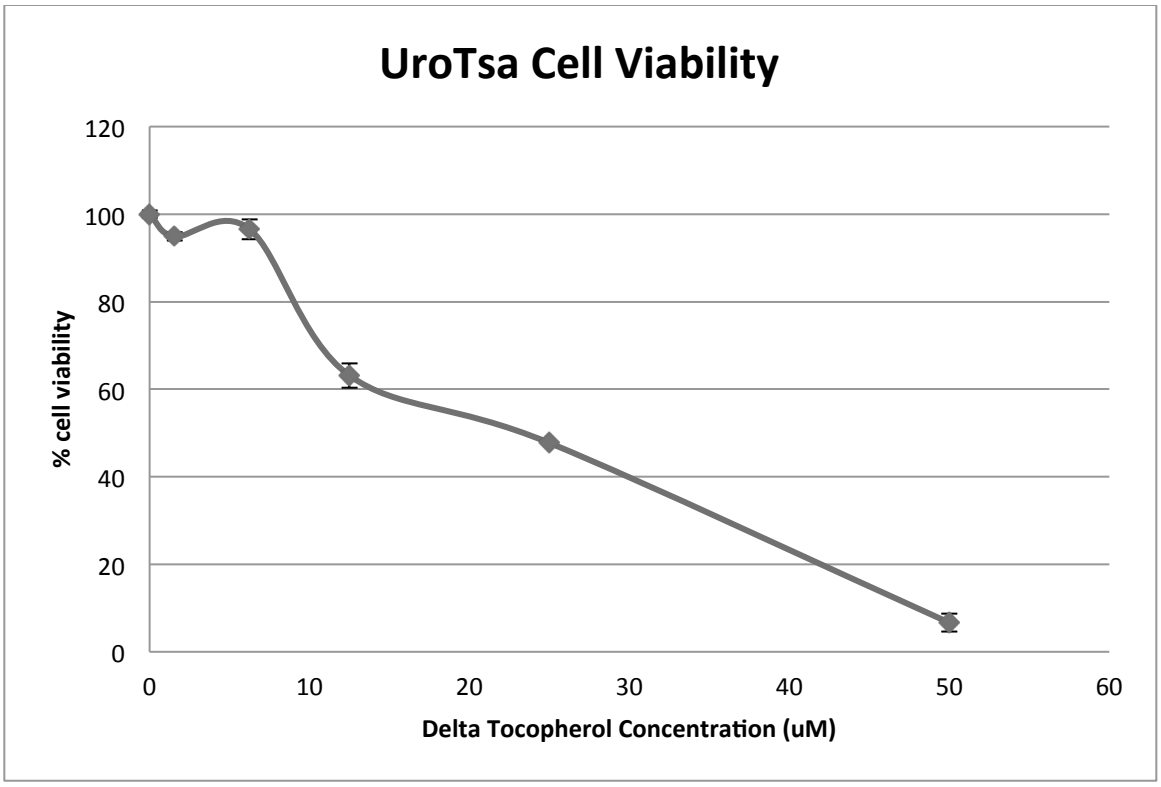

Figure 2.4 Assessment of δ-T cytotoxicity to non-cancerous cells

72-hour treatment of δ-T in healthy immortalized UroTsa cells yielded an IC50 value of 25.46μM.

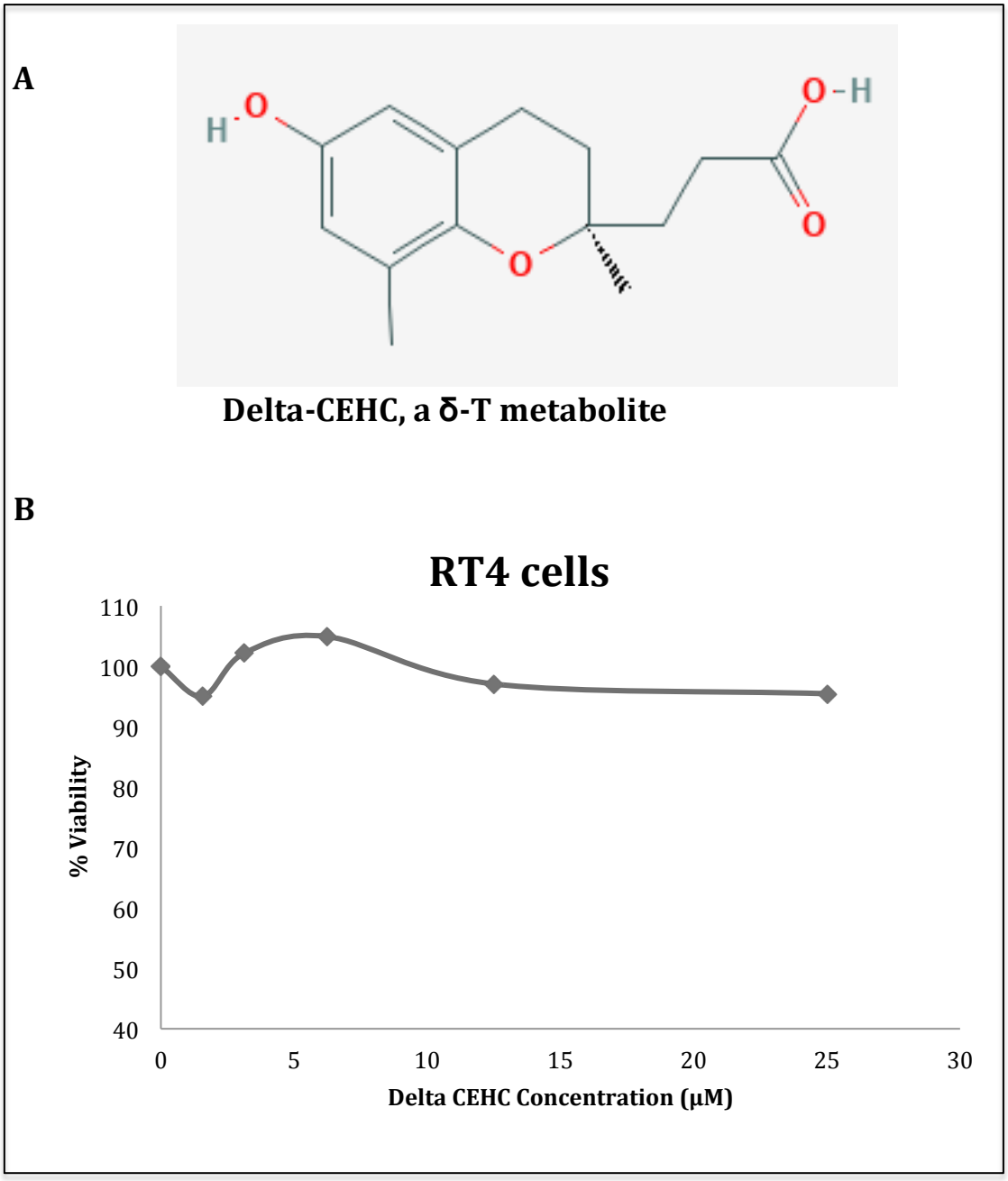

Figure 2.5 Primary urinary metabolite of δ-T, Delta-CEHC shows no cytotoxic effect against BCa cells

A: Structure of Delta-CEHC. B: 72 Hour treatment of RT4 NMIBC cells with Delta-CEHC showed no detectable cytotoxic effect by MTT assay.

Chapter 3: Delta Tocopherol induces UPR-mediated Apoptosis and Autophagy in Bladder Cancer Cells

Introduction

The UPR can be initiated by the ER transmembrane sensor proteins activating transcription factor 6 (ATF6), ER stress sensor and cell fate executor (IRE1), and Protein kinase R (PKR)-like kinase (PERK) under the stressful conditions of glucose deprivation and hypoxia as well as from immune surveillance for cell survival (Chevet et. al., 2015; Wang and Kaufman 2014). All three sensors are triggered when ER protein folding chaperone BiP, which binds the sensor proteins when at rest, dissociates from them to bind to its unfolded protein substrate, allowing the sensor proteins to oligomerize and activate themselves (Lu et. al., 2014). While cells never are truly at rest with regards to BiP activity, stressed cells and cells undergoing rapid division such as cancer cells generally operate under an elevated level of protein folding burden, and rely on the UPR to maintain their regular function.

When initiated in the cell, the UPR can have both pro-survival and pro-apoptotic mechanisms of action that determine the ultimate fate of the cell (Wang and Kaufman 2014). Recently, DR5 was identified as the key point of convergence between these opposing mechanisms which can either become activated leading to apoptosis or downregulated at the mRNA level to allow the cell to survive (Lu et. al., 2014). One ER stress sensor, PERK, generally suppresses translation of existing transcripts after

dissociating from BiP and dimerizing/autophosphorylating, but promotes the translation of transcription factors ATF4 and C/EBP homologous protein (CHOP), which in turn upregulate the expression of DR5 mRNA (Yamaguchi and Wang, 2004). IRE1α, a second UPR sensor, is activated transiently during ER stress (Ron and Hubbard, 2008) (whereas PERK activity persists), and functions anti-apoptotically by inducing DR5 mRNA decay, along with decay of other ER localized RNAs via RIDD (Lu et. al., 2014). Continued detection of stress to the ER without resolution will result in the gradual accumulation of DR5 protein in the cell as the destruction of DR5 mRNA by IRE1α-mediated decay is reduced over time and overcome by its upregulation by the PERK signaling axis. The ER stress-induced accumulation of DR5 causes constitutive activity of the receptor in the absence of Apo2L/TRAIL, its natural ligand (Lu et. al., 2014). DR5 protein accumulation induced by extended stress to the ER results in the initiation of caspase-8 induced apoptosis and cell death (Bellail et. al., 2010).

In addition to the induction of increased folding chaperone expression by the UPR, additional compensatory mechanisms attempt to restore protein homeostasis are triggered by detection of increased unfolded protein load in the ER. Autophagy is a common response to many types of cellular stress that allows cells to clear damaged or nonfunctional organelles and proteins by encapsulating them into large autophagic vesicles in either a targeted or nonspecific fashion (Birgisdottir et al. 2013; Pankiv et al. 2007; Komatsu et al. 2007). Though generally regarded as a pro-survival mechanism, there are situations where autophagy induction appears to have anti-survival outcomes, including the somewhat controversial Autophagic Cell Death pathway

(Kroemer and Levine, 2008) and newly defined situations termed “autosis” which are mediated by Na^+,K^+-ATPase and induce cell death with an autophagy-like phenotype (Liu and Levine 2015). Another mechanism of compensation for ER stress is expansion of the ER regulated by UPR transcription factor XBP1s (Sriburi et al.), which increases cellular space dedicated to protein folding. Selective degradation of excess ER produced in this manner has been observed in yeast in a manner independent of core autophagic machinery which features large vacuole-like autophagic vesicles with ER visible inside when viewed in an electron microscope (Schuck et al. 2014), but there is little evidence for this specific mechanism to date in mammalian cells.

Materials and Methods

Cell Culture: Cell lines were original acquired from ATCC and stored in liquid nitrogen, until thawed and maintained in recommended growth media (RT4, T24-McCoy’s 5A, UMUC3-EMEM, UroTsa-Low glucose DMEM) supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, at 37^o C and 5% CO_2 with normal atmospheric oxygen levels.

Compounds: Tocopherol stock solution was generously provided by Prof. C.S. Yang (Rutgers University) at a concentration of 50 mM. Tocopherols were prepared for use in cell culture by further solubilizing in absolute ethanol before diluting into large volumes of the appropriate complete cell culture media to produce a stock solution for serial dilution.

Lysate Preparation: Cells plated in 10cm tissue culture dishes or 6-well plates were treated for 24 hours with desired concentrations of tocopherols. Following treatment, media was aspirated and cells were washed twice with 1x PBS before incubation on ice with RIPA buffer supplemented with protease inhibitor for 1 hour, after which supernatant containing total cellular protein was collected.

Western Blotting: Lysates were quantified using Lowry assay (Bio-Rad DC protein assay), then standardized concentrations of lysate were prepared with 5x western loading buffer and denatured by heating at 95^{o} C for 10 minutes before loading and running on SDS-polyacrylamide gels (6%-16%, based on target protein size) at 80V for 15 minutes followed by 120V for 1-1.5 hours. Protein was then transferred to a nitrocellulose membrane, blocked for 1 hour in 5% milk TBST, and probed with primary antibody overnight. Membranes were then rinsed in TBST and incubated with secondary antibody for 1 hour, rinsed again, and analyzed on film. Antibodies used were obtained from Cell Signaling Technologies (primary antibodies) and Genetex (tubulin and secondary antibodies).

Flow Cytometry: Cells were plated in 10cm dishes and treated for 24 hours with desired concentrations of δ-T. Cells were then trypsinized and washed with 1x PBS before staining with Annexin V-FITC and PI (BD Annexin V-FITC/PI Apoptosis detection kit). Cells were then analyzed on an Accuri C6 flow cytometer.

DAPI Staining: Cells were plated in 4-well chamber slides for 24 hours prior to treatment, then incubated for an additional 24 hours in the presence of δ-T or vehicle control. The upper chambers were then removed from the slides and the cells fixed in Vectashield DAPI hard-set staining solution and visualized using fluorescence microscopy.

Electron Microscopy: RT4 cells were grown and treated as previously described. Cells were collected after 24 hours of treatment and pelleted to remove media, then fixed in glutaraldehyde and re-pelleted. Pellets were then taken to the UCI Medical Center Pathology Department's Electron Microscopy Core Facility where they underwent Osmium staining and sections were prepared for TEM analysis.

siRNA knockdown: RT4 and UM-UC-3 cells were grown to 70% confluence in 6 well plates, then treated with siRNAs (Qiagen) complexed with Lipofectamine 3000 according to the product protocol. siRNAs used were negative control, and two constructs each targeting DR5 and CHOP, in addition to a sham transfection control. Knockdown of target genes was assessed by western blotting and the more effective siRNA for each target gene was used for further treatments in conjunction with δ-T.

Immunofluorescence staining: T24/LC3-GFP stable Cells were grown in incubation chambers and treated with 3 and 6 μM δ-T for 24 hours. After treatment, the cells were

fixed in 4% formaldehyde in PBS for 30 minutes and washed with PBS twice. The cells were then incubated with blocking and permeabilization solution containing 1% BSA and 0.1% Triton X100 for 1 hour. The cells were probed with anti-Calnexin antibody (an ER marker) diluted in PBS containing 1%BSA + 5% goat serum overnight at 4°C. After washing with PBS three times for 5 minutes, the cells were incubated with secondary fluorescence conjugated antibody for 1 hour. Finally, the cells were washed three more times for 5 minutes with PBS, and mounted with DAPI (nuclear staining) containing Vectashield. Cell images were taken with a Keyence BZ-X700 microscope.

Results

Observation of δ-T treated RT4 cells revealed an autophagy-like phenotype. Confirmation that autophagy was indeed the cause of the phenotypic change observed under electron microscopy was performed using T24-LC3, a bladder cancer cell line stably transfected with GFP-tagged LC3 IIB protein, which is known to aggregate during autophagosome formation. T24-LC3 cells treated with δ-T at varying concentrations demonstrated significant LC3 aggregation consistent with autophagy induction. Additionally, LC3 IIB, Atg5, and Atg12 were found to be upregulated in δ-T treated RT4 and UM-UC-3 cells. **(Figure 3.1)**

To further investigate the observed autophagy-like phenotype in δ-T treated cells, TEM was used to obtain higher magnification and resolution images of RT4 cells treated with 5 μM δ-T. These cells exhibited clear morphological differences from untreated control cells, with both the number and size of autophagic vesicles

dramatically increased in treated cells. Under higher magnification (1100-1650x), the contents of these vesicles appear to be membranous structures closely resembling images of selective ER degradation in yeast (Schuck et al. 2014). The appearance of ER-like structures in the observed autophagosomes was further examined by using immunofluorescent staining for ER marker Calnexin in T24-LC3 cells treated with δ-T. Colocalization of red (Calnexin) staining and GFP-tagged LC3 aggregates in δ-T treated cells further reinforced the hypothesis that ER components were degraded in the autophagosomes. **(Figure 3.2)**

As autophagy is generally considered a pro-survival response, and the autophagy phenotype observed previously closely resembled and ER-stress induced form of autophagy, I next tested for apoptosis induction, as this is the primary mechanism of cell death induced by ER stress. An apoptosis assay **(Figure 3.3)** using flow cytometry revealed a strong dose-dependent induction of apoptosis in δ-T treated cells. DAPI staining of 24-hour δ-T treated RT4 cells for revealed visible nuclear fragmentation and morphological changes consistent with apoptotic induction. **(Figure 3.4)**

RT4 and UM-UC-3 cells treated overnight with δ-T were lysed as described, and western blotting revealed increased DR5 expression and corresponding increases cleavage of caspases 3 and 8, further reinforcing evidence of apoptotic cell death induction **(Figure 3.5)**. siRNA knockdown of DR5 was performed as described, and cells treated for protein lysate collection. Western blotting of DR5 knockdown cells revealed a moderate reduction in cleavage of caspase 3 and PARP. **(Figure 3.6)**

To investigate the cause of δ-T induced DR5 upregulation, further western blotting was performed on treated cell lysates, revealing upregulation and phosphorylation (activation) of ER stress sensors PERK and IRE1α, as well as upregulation of protein folding chaperone BiP, and UPR induced transcription factor CHOP. **(Figure 3.7)**

siRNA knockdown of CHOP in RT4 cells was performed next to determine if suppression of the ATF4/PERK/CHOP pathway would attenuate the cytotoxic effects of δ-T treatment. After 24 hours of δ-T treatment at 2.5 μM and 5 μM concentrations, CHOP knockdown cells showed only a small loss of viability compared to the untreated CHOP knockdown control cells as measured by flow cytometry (6.8% viability reduction at 2.5 μM δ-T, p=.005; 4.43% viability reduction at 5 μM δ-T, p=.0267) whereas cells transfected with negative control siRNA and treated with 2.5 μM or 5 μM δ-T were 10.067% (p=.0001) and 22.23% (p<.0001) less viable than untreated negative control cells, respectively. **(Figure 3.8)**

Discussion

After observing the dramatic difference in the anti-cancer activities of the different tocopherol isoforms, determining the mechanism by which δ–T exerts its cytotoxic effect on BCa cells became the next logical step. As antioxidant activity is the most frequently considered function on Vitamin E in the diet, I began by assessing the three tested tocopherol isoforms in an ROS assay, however no significant difference was detected.

The observed morphological changes in treated cells, particularly the formation of large vacuole-like structures suspected to be large autophagic vesicles, prompted me to investigate the potential involvement of autophagy in δ–T's mechanism of action. In order to obtain better images of the observed morphological changes, RT4 cells were treated and Osmium stained for transmission electron microscopy (TEM) imaging. The resulting images showed distinct differences in cell morphology between the treated and control groups, with δ–T treated cells displaying significantly more frequent formation of vacuole-like bodies and a much larger average size of these bodies in the cells in which they appeared. Further support for the hypothesis that these bodies were indeed autophagic vesicles came in the form of upregulation of LC3B and other components of autophagic machinery identified by western blotting of treated cell lysates and observation by fluorescence microscopy of LC3 aggregate formation consistent with autophagy induction in T24-LC3 cells expressing GFP-tagged LC3B protein and treated with δ-T.

Examination of the highest magnification (4400x) TEM images also revealed unknown membranous structures within the autophagic vesicles observed in δ–T treated cells. A search of available TEM images found in literature of autophagic vesicles yielded a surprising similarity between the observed structures in treated human cancer cells and the autophagic phenotype exhibited in *Saccharomyces cerevisiae* (yeast) cells which had been treated with ER-stress inducing compounds (Schuck et al. 2014). Furthermore, the membranous structures shown in **Figure 3.2** closely resembled structures which had in yeast autophagosomes been identified as

"ER-whorls," the result of ER expansion in response to drug-induced stress (Bernales et al. 2006). The selective degradation of ER, including ER-whorls, was demonstrated in this study to occur as part of the autophagic response to ER stress and has not been similarly described in humans (Schuck et al. 2014).

To establish whether or not the observed structures in TEM images were indeed the result of autophagic ER degradation, we again employed the T24-LC3 cell line to take advantage of its GFP tagged LC3, which allows easy identification of autophagosome formation induced by δ-T treatment. δ-T treated cells were subjected to immunofluorescent staining using anti-Calnexin primary antibody, an ER-specific marker and component of the UPR. **Figure 3.2** shows the appearance of calnexin staining colocalized with GFP-LC3 aggregates that supports the hypothesis that ER degradation is occurring in δ-T treated cells. In combination with the results of other studies that showed ER-stress induced reticulophagy in a yeast model (Schuck et al. 2014), these observations suggest that a similar mechanism may indeed be occurring in δ-T treated bladder cancer cells, though to what extent this phenomenon is responsible for δ-T's cytotoxic effects remains unclear, as we later found strong evidence for the canonical induction of apoptosis by the UPR as the primary driver of δ-T induced cell death. Nonetheless, observation of this phenomenon in mammalian cells remains a novel finding that may ultimately have strong implications for the study of autophagy's role in mammalian stress-induced cell death and potential for investigation as a novel mechanism for therapeutic targeting.

Due to the link between the observed autophagic phenotype and ER stress, I next examined whether apoptosis induction was occurring in δ–T treated cells, as this mechanism provides the primary cell death endpoint for the UPR. To that end, the next step in determining the mechanism of cell death caused by δ–T was to use flow cytometry to analyze apoptotic markers, which revealed a strong dose-dependent increase in apoptosis induction and informed the next experimental choices. δ–T treated cells were also stained with DAPI, which revealed nuclear fragmentation consistent with apoptotic cell death in addition to the other observed morphological changes.

Review of existing literature supports an ER stress related mechanism of apoptotic cell death, as detected in other studies focusing primarily on γ-T or tocotrienols (Lu et. al., 2010; Li et. al., 2011; Lambert et. al., 2009; Ju et. al., 2009; Lee et. al., 2009). To assess various molecular pathways of cell death induction by δ–T, RT4 and UM-UC-3 cell lines (chosen for their response to δ–T treatment and as representative models of both NMIBC and MIBC, respectively) were dosed with δ–T at 3μM and 6μM for 24 hours and then lysed and protein lysates collected. These lysates were analyzed by western blotting and revealed increased expression of DR5 in δ–T treated cell lysates.

After observing the DR5 upregulation in δ–T treated cells, I tested the downstream results of DR5 activity and found increased caspase 8 cleavage in δ–T treated cells consistent with activation of the extrinsic apoptosis pathway (Li et al. 2008, McIlwain et al. 2013) and a corresponding increase in caspase 3 cleavage. siRNA

knockdown of DR5 attenuated the effects of δ–T treatment, reducing both caspase 3 and PARP cleavage levels in anti-DR5 siRNA transfected cells versus negative control siRNA transfected cells. Knockdown of CHOP, a directly upstream transcription factor for DR5, nearly eliminated the sensitivity of RT4 cells to δ-T treatment, with knockdown cells losing only 4.43% viability at 5 μM δ-T versus a 22.23% viability loss in cells treated with negative control siRNA and 5 μM δ-T.

I next looked for changes in the expression of ER stress and UPR markers in the previously examined lysates. In both cell lines, δ–T treatment strongly increased expression of UPR components. PERK and IRE1α both showed increasing phosphorylation shifts consistent with activation in response to ER stress. Protein folding chaperone BiP expression was similarly increased, as was the expression of ATF4 and CHOP, indicating that the pro-apoptotic accumulation of CHOP in response to extended and unresolved ER stress (Yamaguchi and Wang, 2004) was indeed occurring in response to δ–T treatment, consistent with the previously observed caspase activation. These findings provided strong evidence of DR5 mediated apoptosis in response to δ–T treatment as the primary mechanism by which BCa cells are killed by δ-T.

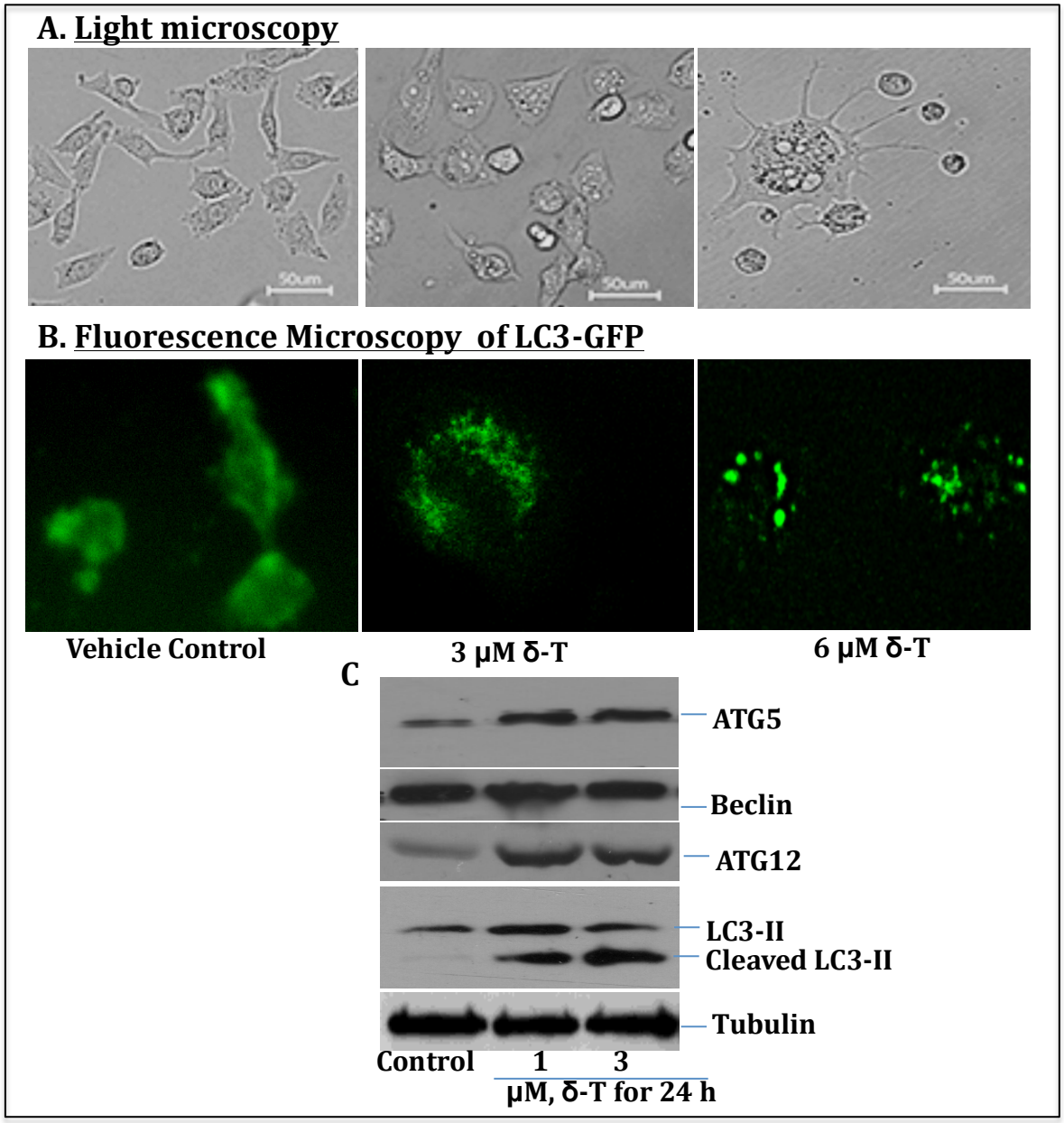

Figure 3.1 Autophagy induction in δ-T treated cells

A, C: RT4 cells treated for 24 hours with δ-T show autophagy like phenotype and upregulation of autophagy related genes. B: T24-LC3 cells expressing GFP-tagged LC3B protein exhibit LC3 aggregation after 24 hour treatment with δ-T.

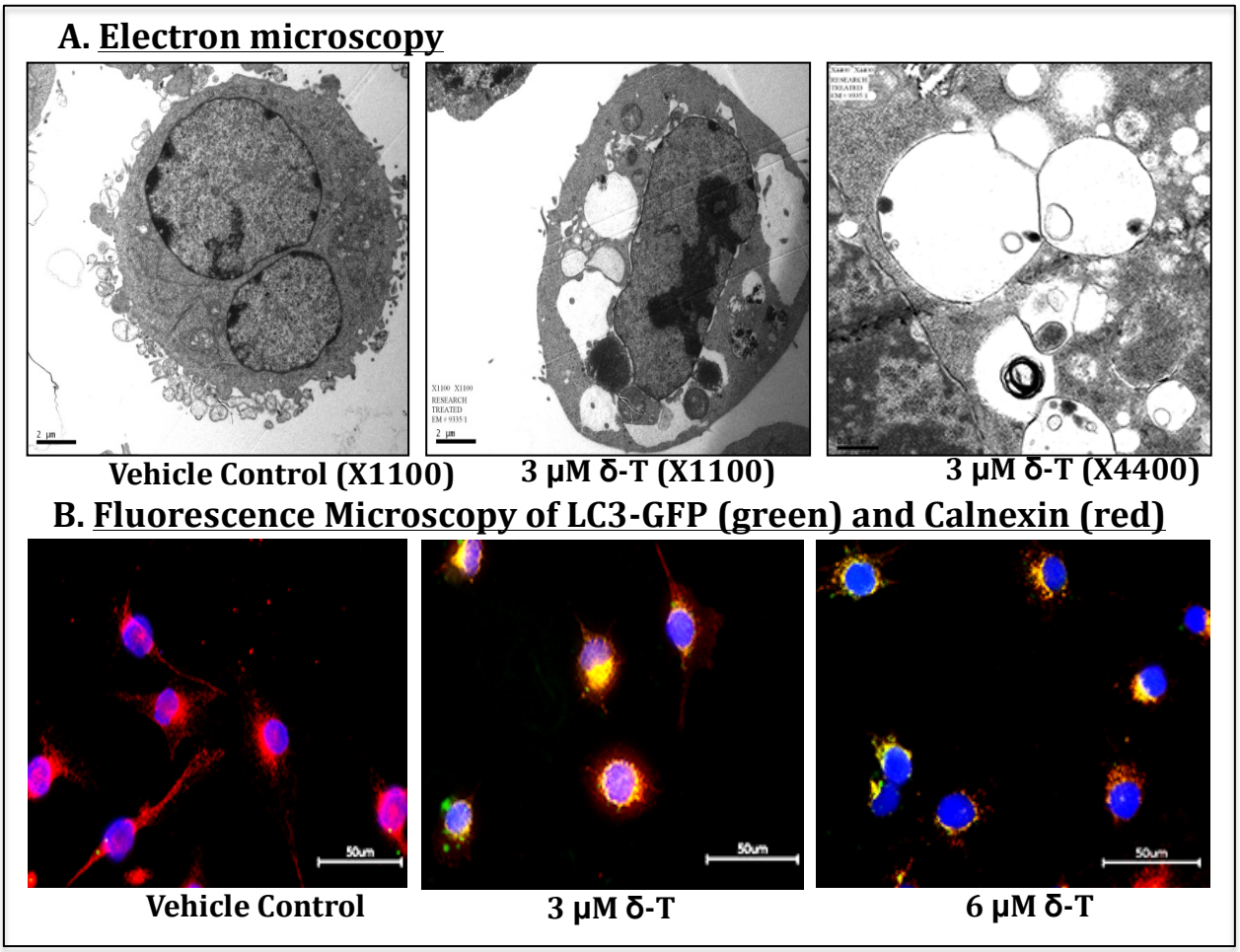

Figure 3.2 Delta Tocopherol treatment induces ER degradation by autophagy

A: Electron microscopy images of RT4 cells treated with δ-T for 24 hours reveals formation of large autophagosomes and their ER membrane-like contents.

B: Immunofluorescence staining for Calnexin (red), LC3-GFP (green), and DAPI (blue) shows colocalization of ER and autophagosomes.

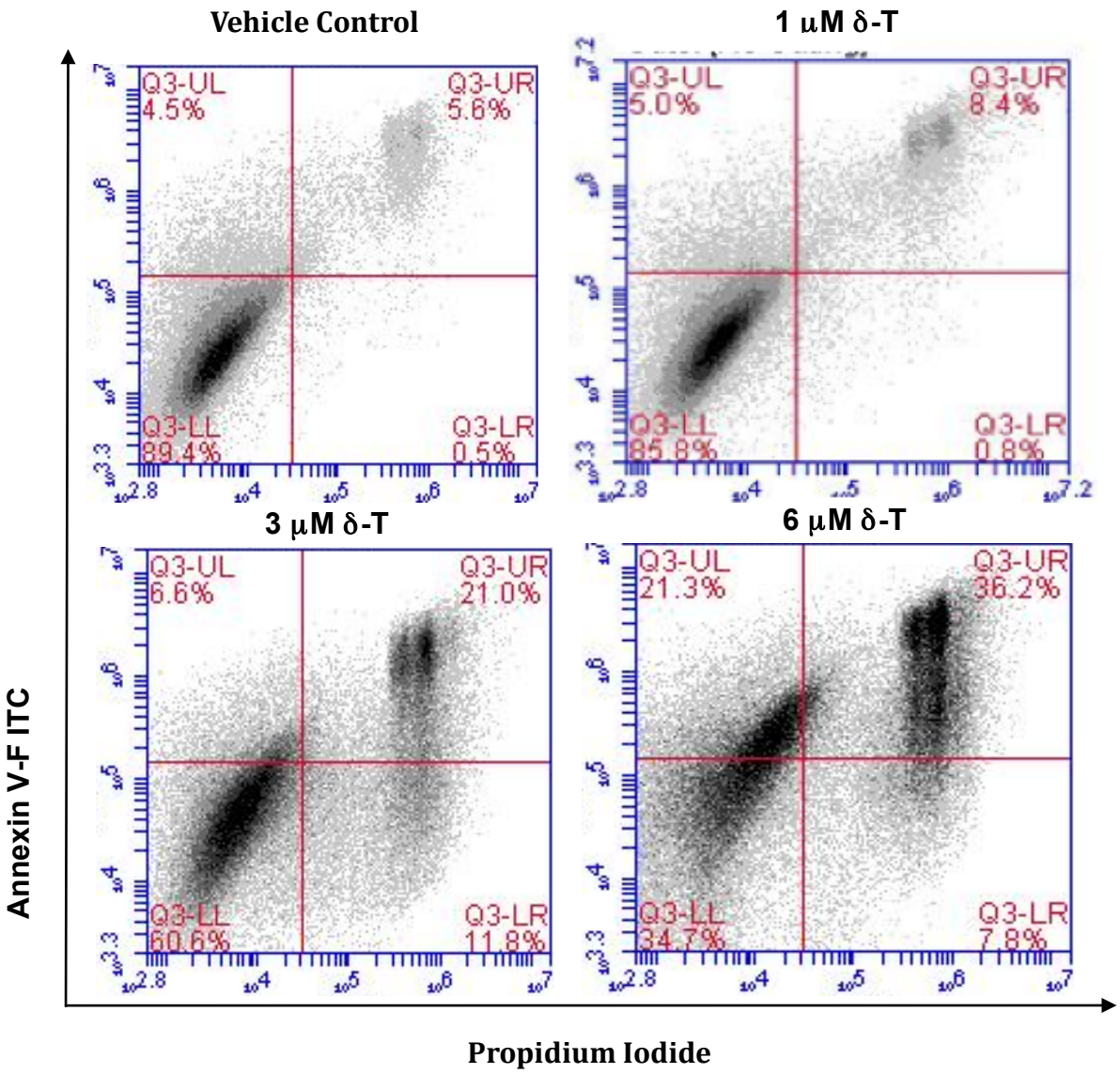

Figure 3.3 Delta Tocopherol treatment induces a dose dependent increase in apoptosis in RT4 bladder cancer cells

RT4 cells were treated for 24 hours with indicated concentrations of δ-tocopherol. The percentage of cells in the upper right quadrant represents apoptotic cells.

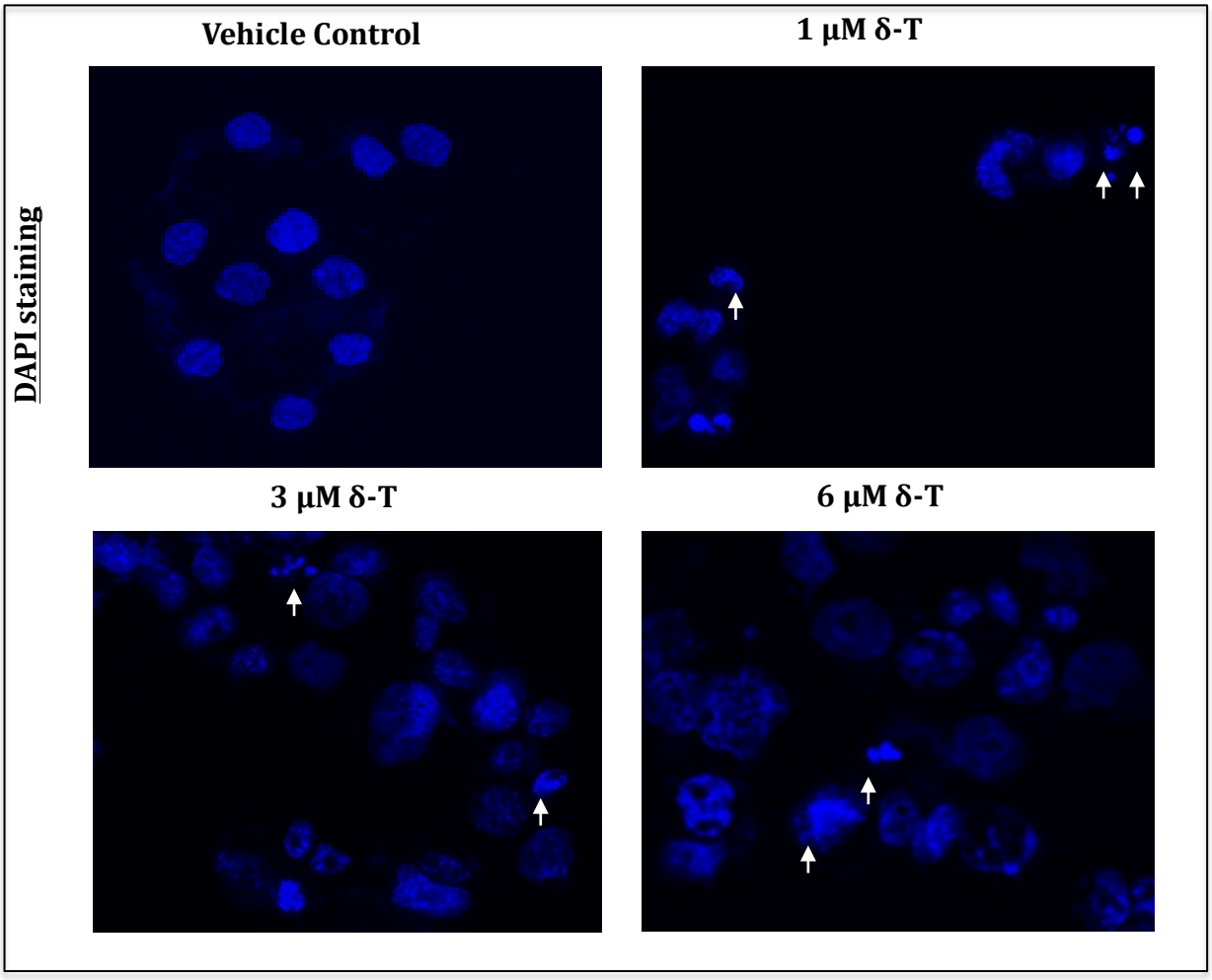

Figure 3.4 DAPI staining of RT4 cells treated with δ-T exhibits characteristics of apoptosis

DAPI stained images of δ-T treated RT4 cells exhibit nuclear fragmentation characteristic of apoptosis (indicated by white arrows.)

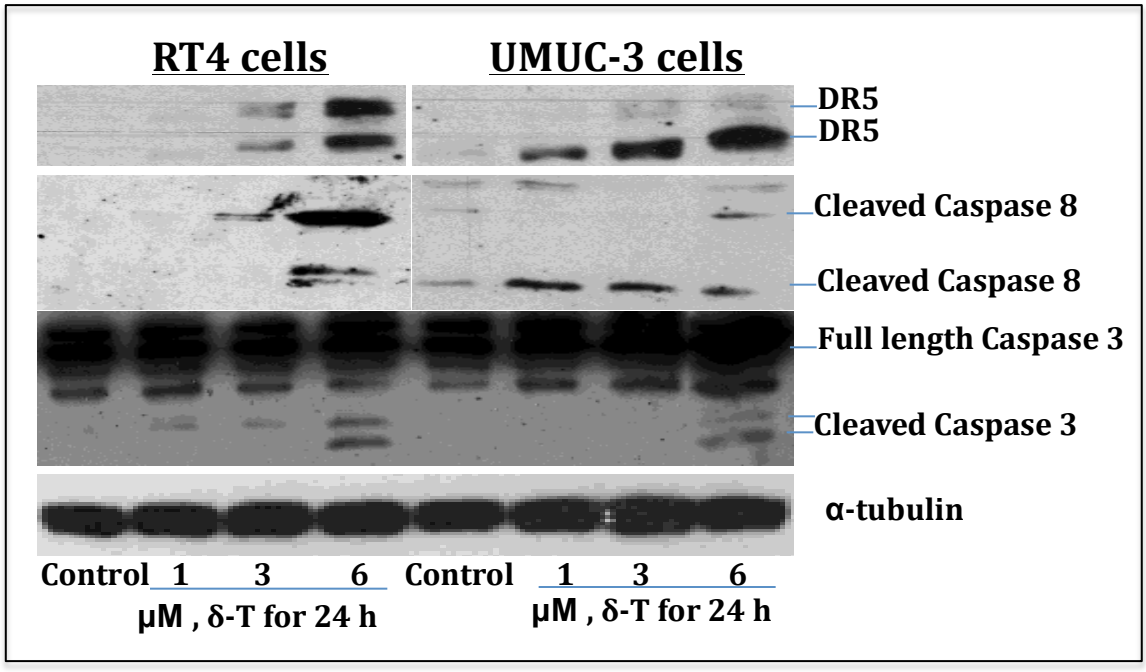

Figure 3.5 Western blotting of δ-T treated cells

Cells treated with vehicle control or 1μM , 3μM , or 6μM δ-T for 24 hours were lysed and analyzed by western blotting, revealing DR5 upregulation and apoptosis induction in treated cells.

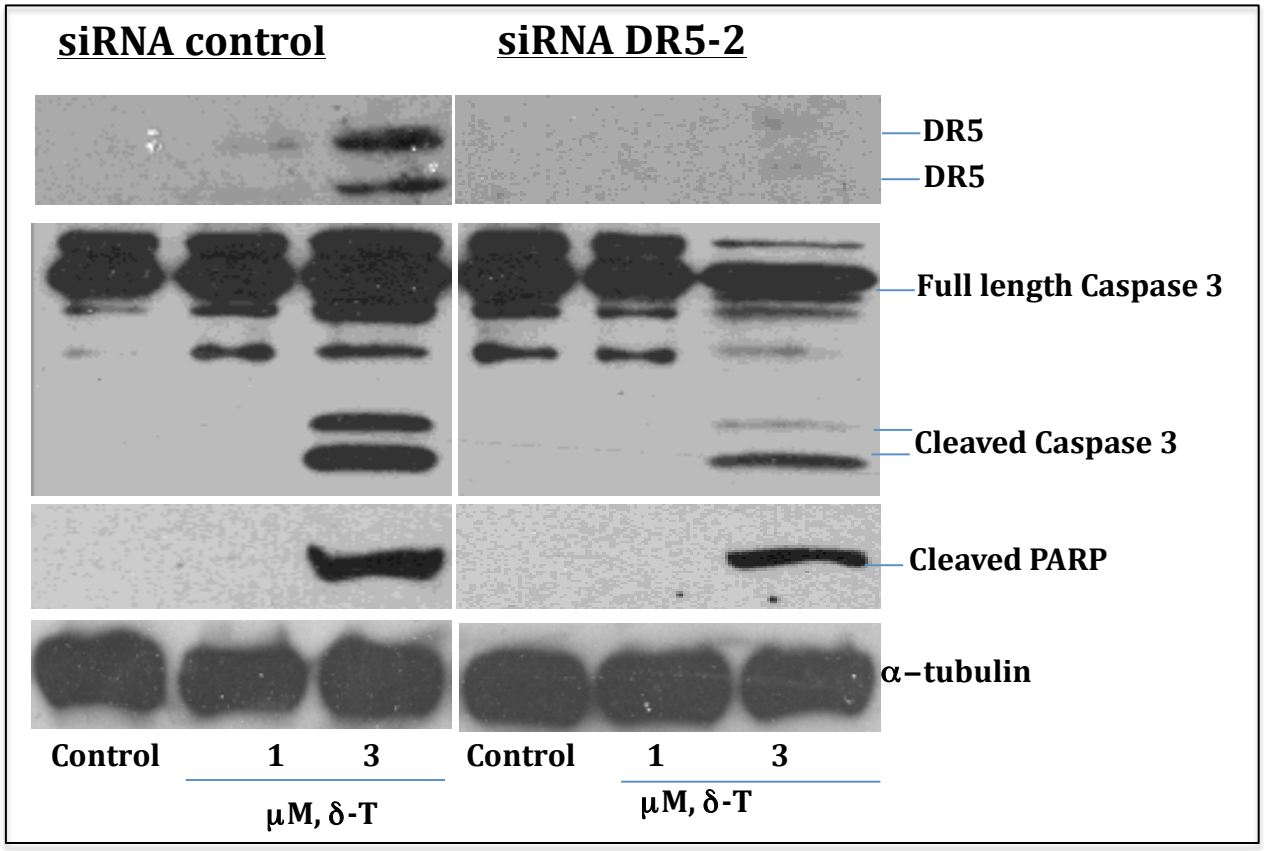

Figure 3.6 siRNA knockdown of DR5 reduces markers of apoptosis induction

siRNA knockdown of DR5 reduced caspase 3 and PARP cleavage in δ-T treated cells.

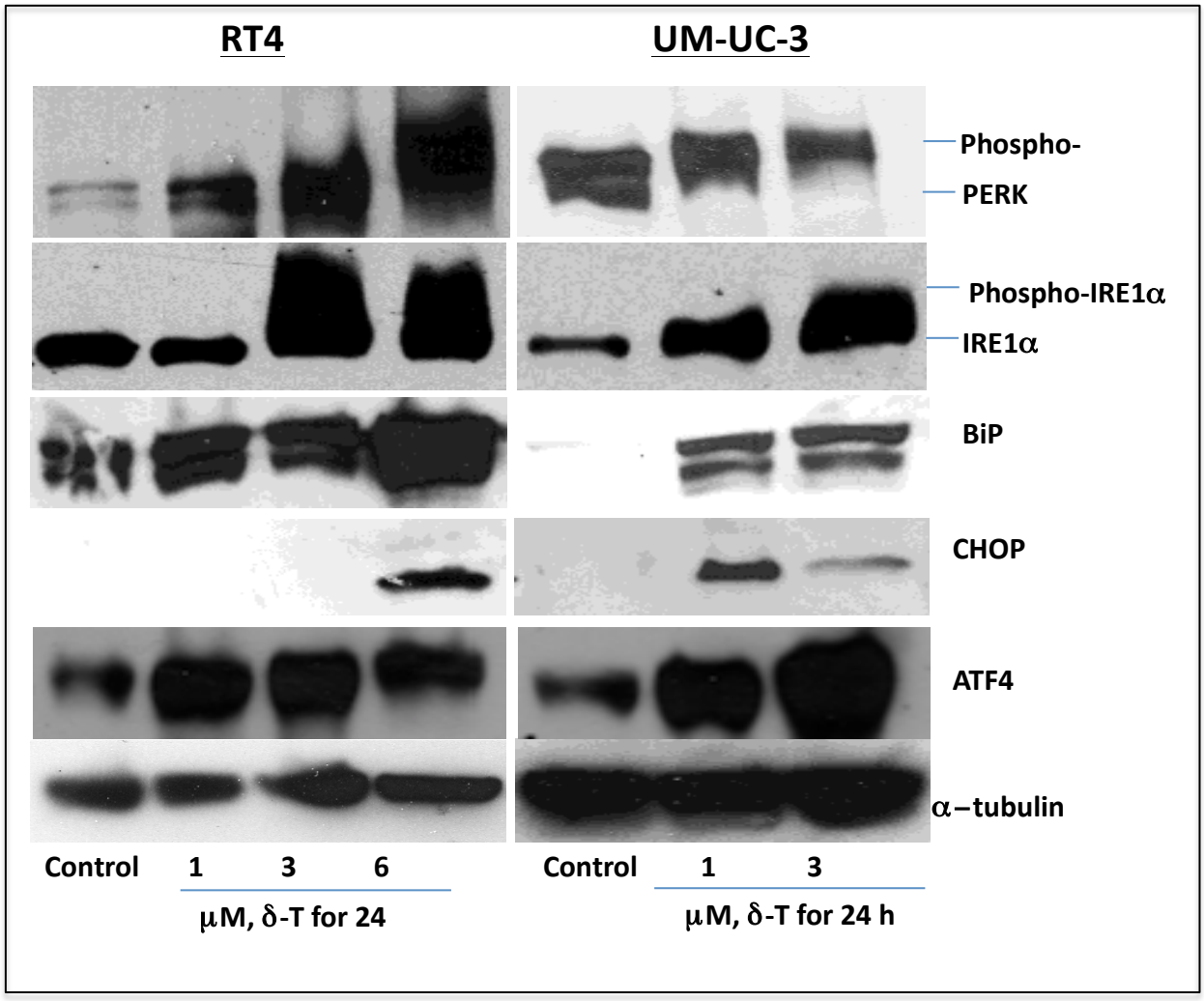

Figure 3.7 δ-T treatment induces UPR activation

Western blotting of lysates from RT4 and UM-UC-3 cells treated with δ-T revealed upregulation and phosphorylation of ER stress sensors PERK and IRE1α, upregulation of protein folding chaperone BiP, and upregulation of downstream transcription factors ATF4 and CHOP.

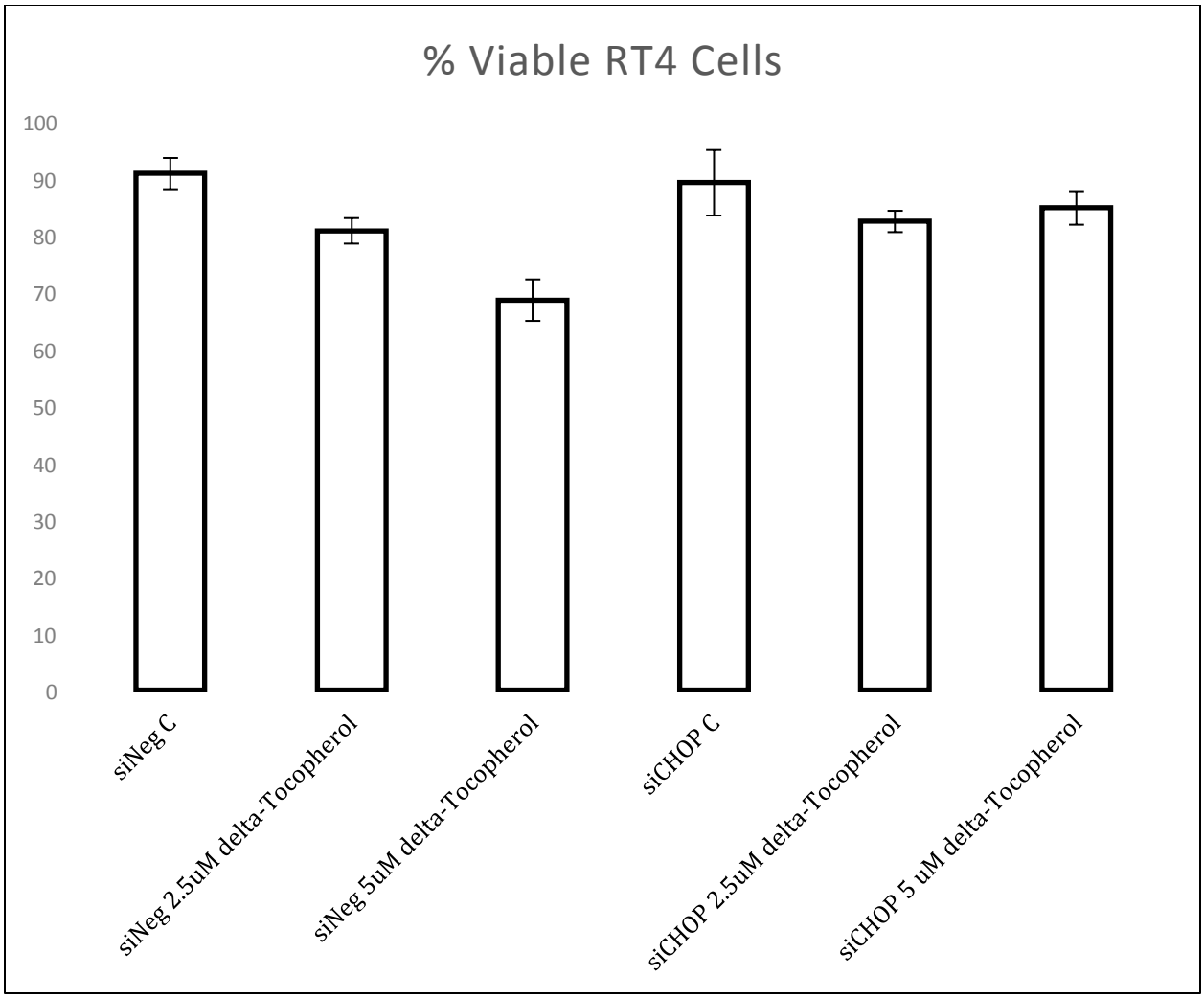

Figure 3.8 CHOP knockdown reduces sensitivity to δ-T treatment

RT4 cells were treated with negative control siRNA or CHOP targeted siRNA. CHOP knockdown cells showed only a small loss of viability compared to the untreated CHOP knockdown control cells as measured by flow cytometry (6.8% viability reduction at 2.5 μM δ-T, p=.005; 4.43% viability reduction at 5 μM δ-T, p=.0267). Cells transfected with negative control siRNA and treated with 2.5 μM or 5 μM δ-T were 10.067% (p=.0001) and 22.23% (p<.0001) less viable than untreated negative control cells, respectively.

Chapter 4: Dietary Delta Tocopherol Administration Significantly Reduces Tumor Growth and Initiation in Mouse Models of Bladder Cancer

Introduction

As natural compounds commonly occurring in the diet, tocopherols are attractive candidates for development as non-toxic clinical therapeutic agents. While Vitamin E is a fat-soluble mixture of compounds that may cause toxicity at high doses, a tolerable upper intake level of up to 1000mg per day exists at which no likely health risks are posed to humans (Institute of Medicine. Food and Nutrition Board, 2000). Vitamin E supplements which have demonstrated widely varying effects in clinical cancer studies (Smith-Warner et. al., 2003; Ju et. al., 2010; Mahabir et. al., 2008; Lippman et. al., 2009; Klein et. al., 2011; Lee et. al., 2005; Wu et. al., 2014) consisted primarily of α tocopherol, which we have demonstrated to be ineffective for the purposes of bladder cancer therapeutics. Additionally, studies have demonstrated that the co-administration of mixed tocopherols results in the predominance of the non-chemopreventive α-tocopherol in the bloodstream at the expense of the serum concentrations of the more effective γ- and δ –Ts (Huang and Appel, 2003). This phenomenon is caused by the high affinity for α-T of the aptly named α-tocopherol transport protein, which protects its substrate from degradation in the liver and is competitively inhibited from protecting other tocopherols by the presence of α-T

(Huang and Appel, 2003). The administration of mixed or primarily a-T vitamin E supplements therefore may actually have an overall negative effect on the levels of the more beneficial γ- and δ–Ts (Huang and Appel, 2003) which may be responsible for the findings in those studies which showed increased cancer risk or incidence resulting from vitamin E supplementation (Klein et. al., 2011). Study of δ-T alone is therefore necessary to demonstrate the potential for further development of δ-T as a novel chemopreventive or therapeutic agent and to eliminate the effect of supra-nutritional α-T intake on bioavailable δ-T levels. Dietary supplementation of δ-T, if effective, would provide an easy means of administration that could improve the efficacy of existing treatments or potentially even be used as an independent chemopreventive approach for lower risk BCa patients.

In order to evaluate the *in vivo* efficacy of δ-T supplementation against BCa, two mouse models were be used answer several experimental questions important to justify the further pursuit of δ-T as a chemopreventive agent. Both mouse models gave valuable safety information about δ-T supplementation and provide evidence of the oral bioavailability of δ-T, which will significantly improve the ease of δ-T's eventual use in patient populations. A transgenic model of bladder tumorigenesis, which mimics the progression of bladder cancer in human patients, was previously employed by our group to pre-clinically evaluate chemopreventive drugs (Liu et al., 2016) and was again employed to demonstrate effectiveness of dietary δ-T at the interior urothelial lining. Additionally, a xenograft model employing MIBC cell line UM-UC-3 was used to establish whether δ-T would effectively reach distant tumor sites via the bloodstream

and to account for any unexpected effects that may have been particular to the transgenic model employed earlier.

Materials and Methods

UPII Mutant Ha-ras model: Heterozygous UPII-mutant Ha-ras+/− females were cross-bred with heterozygous UPII-mutant Ha-ras+/− males, and offspring were genotyped to select homozygous UPII-mutant Ha-ras mice.

Chemoprevention study: Male homozygous UPII-mutant Ha-ras mice were randomly assigned to groups and provided with control diet or diet supplemented with 0.2% w/v δ-T, beginning at the age of 30 days and ending at the age of 180 days. The bladder, ureter, and kidneys of survived mice were fixed in 10% neutral-buffered formalin and processed for paraffin embedding, sectioning, and staining by hematoxylin and eosin (H&E). Sections of each urinary bladder tumor, ureter, and kidney were histologically evaluated by a pathologist blinded to the experimental groups. Control and δ-T supplemented diets were prepared commercially (Dyets, Inc.). Mice were permitted free access to food and water. All animals were examined daily for morbidity, mortality, clinical signs of ataxia, and toxicologic effects including respiratory depression, any neurobehavioral abnormalities, discoloration of the skin and eyes, and altered motor activity. Food intake and body weight were recorded biweekly. Animal care and treatments performed were in accordance with Institutional guidelines and the approved IRB protocol UCI #2004-2540. At the end of treatment, all surviving mice

were euthanized by CO_2 asphyxiation. Serum samples were obtained by cardiac puncture, and urine samples were obtained by bladder massage. A laparotomy was conducted to expose all major organs, which were then inspected for frank toxicity and any visible abnormalities. Photographs were taken to document the animals and their urogenital systems in situ. All non-bladder organs were removed and fixed in formalin for standard hematoxylin and eosin (H&E) slide preparation and examination. Any evidence of edema, abnormal organ size, or other abnormalities in non-bladder organs was noted. A portion of the bladder was fixed in 10% neutral-buffered formalin for histopathologic evaluation and the rest was snap frozen in liquid nitrogen and stored at −80°C for future analysis.

Xenograft model: UM-UC-3 BCa cells were cultured and collected as described previously. Cells were then resuspended in BD Matrigel at $1x10^7$ cells/mL, and 1 million cells/animal were injected subcutaneously into the right flank of female nude mice (n=10). After tumors were allowed to initiate for 1 week, mice were divided into control (n=5) and δ-T (n=5) groups with comparable initial tumor sizes and body weights. 0.2% w/v δ-T supplemented diet or vehicle control diet was provided to the appropriate groups for at-will feeding, and food intake, water intake, and tumor size were measured bi-weekly. Animal care and treatments were conducted in accordance with Institutional guidelines and the approved UCI IRB protocol #2004-2540. Mice were monitored to ensure a humane endpoint and euthanized by CO_2 asphyxiation, subsequently tumors were excised, weighed, and portions of each tumor fixed in

formalin or snap-frozen. Formalin fixed samples were then paraffin-embedded for immunohistochemical analysis.

Immunohistochemistry: Paraffin-embedded sections (5-μm thick) were heat immobilized, deparaffinized using xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. The sections were then incubated with mouse monoclonal anti-Ki-67 antibody (Abcam, 1:800) for 1 hour at 37°C in a humidity chamber. Isotypic IgG was used as a negative control. Sections were incubated with biotinylated rabbit anti-mouse IgG (1:200 in 10% normal goat serum) for 30 minutes at ambient temperature. The sections were then incubated with 3, 3′-diaminobenzidine (DAB) following the R&D Systems Cell & Tissue Staining Kit protocol. The sections were finally counterstained with diluted Harris hematoxylin (Sigma Chemical Co.) for 2 minutes, and rinsed in Scott water. Proliferating cells were quantified by counting the Ki67-positive cells and the total number of cells using the automated cell counting feature of the BZ-X analyzer software (Keyence) in three high powered fields selected from three different tumors per group.

Results

UMUC-3 Xenograft Model

Female nude mice injected subcutaneously with $1x10^6$ UMUC-3 cells were placed into control (n=5) and δ-T fed (n=5) groups with comparable initial body weights and tumor sizes one week after tumor implantation. Each group was provided with appropriate diet (Vehicle control or 0.2% w/v δ-T supplemented diet) in unlimited quantity. Mice were monitored bi-weekly for changes in tumor size, overall body weight, and food/water intake. No changes in behavior were noted in the mice over the course of the experiment. Overall body weight,, as well as food and water intake over the course of the experiment, were not found to differ significantly between the control and δ-T fed groups. Notably, the endpoint of this experiment was determined by the health of mice in the control group, as the mice in the δ-T fed group remained healthy and active at the end of the second week, but experiment could no longer be humanely continued due to the burdens placed on the health of the mice in the control group by their large tumor sizes. After 2 weeks of treatment, mice were euthanized by CO_2 asphyxiation, weighed, and their tumors removed, photographed **(Figure 4.1)**, and weighed. Mice in the δ-T fed group exhibited significantly smaller tumors than those in the control group, with an average 58.6% (p=.000434) lower tumor mass with no change in body weight. **(Figure 4.2)** Upon necropsy, and throughout the course of the experiment, no indications of toxicity or any other side effects were observed in either group.

Tumors excised from control and δ-T fed groups were fixed in formalin and embedded in paraffin, sectioned, then stained with H&E and for Ki-67 and mounted on slides. Tumors from δ-T fed mice showed significantly lower (7.14% reduction in positive cells, p=.0058) Ki-67 staining than control mouse tumors, as determined by automated cell counting of 40x fields from three separate tumors, indicating reduction in overall cell proliferation rate. **(Figure 4.3)**

UPII Mutant Ha-Ras chemoprevention model

Homozygous male UPII mutant Ha-ras transgenic mice were genotyped and randomized into groups fed with vehicle control diet (n=20) or diet supplemented with 0.2% w/v δ-T (n=18) for 150 days. The δ-T diet significantly decreased the mean bladder weights (a surrogate for tumor weight) of survived Ha-ras mutant mice by 56% (control vs δ-T, 0.259 ± 0.015 gram vs. 0.114 ± 0.012 gram, P<0.0001) **(Figure 4.4)** with no significant difference in overall body mass and no detectable indication of toxicity. Treated mice exhibited smaller bladder size upon necropsy and reduction in observable tumor initiation in H&E treated bladder sections. **(Figure 4.5)** The survival rate at 150 days of control diet fed mice was also lower than the survival rate for δ-T fed mice (30% control, 44.4% δ–T fed), though this difference fell just short of statistical significance (p=.0895). **(Figure 4.6)** Body weights remained comparable between vehicle control and δ–T fed groups over the course of the experiment, though δ–T fed mice showed a slight reduction in food consumption. **(Figure 4.7)**

Discussion

When evaluating the potential of any new chemotherapeutic or chemopreventive agent, it becomes necessary to determine if the *in vitro* observed activities are preserved *in vivo*. This process is particularly important when investigating a compound with potential for use as a dietarily supplemented agent, such as δ–T. The pharmacokinetic properties of δ–T, in particular with regards to the interference with its bioavailability caused by αTTP's higher affinity for α-T (Brigelius-Flohe and Traber 1999, Traber 2007), demand the application of δ–T as an individual dietary agent rather than merely as a component of dietary Vitamin E intake.

Two mouse models were used to evaluate the efficacy of dietary δ-T. The UPII mutant Ha-ras transgenic mouse model mimics the initiation of human papillary transitional urothelial cell carcinoma (UCC) in both pathology and molecular pathways (Mo et. al., 2007; Wu 2005). UPII mutant Ha-ras transgenic mice develop urothelial hyperplasia and non-muscle invasive papillary UCC sequentially within 6 months of age (Mo et. al., 2007; Wu 2005). Tumors can be observed in the bladder, the renal pelvis and the ureters. This model is ideal for testing the pre-clinical efficacy of agents in preventing and treating the recurrence and progression of NMIBC, and has been used by our group successfully in the past (Liu et al. 2016). As a surrogate measurement for tumor burden, the bladder weight is measured for each mouse. As tumor initiation in this model happens in the same physiological location as BCa initiation in humans, this model also provides evidence of δ-T activity at the most important sites for its chemopreventive effects to be beneficial.

To establish a xenograft model using human cancer cells, which would allow any issues specific to our transgenic model to be eliminated and demonstrate bioavailability of δ-T through the bloodstream, UM-UC-3 cells were cultured as previously described, then collected and resuspended in BD matrigel. The cell suspension was then injected subcutaneously in the right flank of nude mice, and tumors were allowed to establish themselves and grow for one week before initiation of dietary δ tocopherol feeding and tumor growth monitoring. The MIBC cell line UM-UC-3 was chosen for this study as MIBC cells are more representative of cancer cells that would be found in distant tumor sites than a NMIBC cell line such as RT4 would be. This model also allowed for direct measurement of tumor size over the course of the experiment, as well as access to whole tumors after the experimental endpoint.

In both experimental models, mice were sorted into the control or experimental groups with comparable initial body weights and by comparable measurable tumor size in the xenograft model. Mice in both models were supplied with unlimited quantities of feed supplemented with 0.2% (w/v) δ-T or control food, and the overall weight of each animal and the food intake per cage were monitored bi-weekly over the course of the experiment. Tumor growth in the xenograft experiment was measured bi-weekly over the course of the experiment by physical measurement of the tumor with calipers. The endpoint of the xenograft experiment was determined at the point when the tumor size of mice in the control diet-fed group became too large to humanely continue the experiment. All mice in the δ-T fed group remained healthy and exhibited visibly smaller tumors at this time point, and treatment could feasibly have been continued at

the expense of consistency with the control group. However, to maintain comparable data both groups of mice were sacrificed and their tumors excised, weighed, and photographed. Tumor samples were then fixed, sectioned, and stained for IHC analysis.

With an obvious visible difference in tumor size **(Figure 4.1)** and 58.6% lower tumor mass at comparable body weight **(Figure 4.2)**, the δ-T fed mice fared significantly better than their control counterparts over the time course of the experiment. Tumor samples treated with Ki-67 as a marker of cell proliferation revealed that δ-T treated mouse tumors had significantly reduced (16.88% δ-T fed vs. 24.02% control, average of randomly selected 40x fields of three different tumors per group) cell proliferation. **(Figures 4.3)**

Transgenic mice were treated for a much longer time course than xenograft mice (150 days vs. 14 days) as a model of tumorigenesis and to evaluate the potential chemopreventive effects of dietary δ-T. While the 14.4% improvement in survival rate at 150 days observed in the δ-T fed group did not reach statistical significance, this may be a reflection of cohort size and follow-up time rather than poor efficacy on the part of the treatment. Further experiments with increased sample size and more dose levels of dietary δ-T are needed. In the survived mice a significant 56% average reduction in overall bladder weight, a surrogate measurement for tumor burden, was measured in δ-T fed mice vs. vehicle control fed mice **(Figure 4.4)**, accompanied by a visible decrease in genitourinary tract enlargement and reduction in hyperplasia and papillary tumors observable in H&E stained bladder sections. **(Figure 4.5)** The observed 14.4% lower survival rate of vehicle control fed mice vs. δ-T fed mice fell short of statistical

significance (p=.0895), however a longer follow up time or sample size could likely improve this result. **(Figure 4.6)** δ-T fed mice had a slightly lower average food consumption than mice fed with vehicle control diet, though overall body weight did not vary significantly. **(Figure 4.7)**

The strong effect observed in both mouse models is indicative of significant potential for the application of dietary δ-tocopherol as a cancer preventative or therapeutic agent. The significant reduction in size and mass of subcutaneously implanted tumors demonstrates the presence of sufficient δ tocopherol levels in the bloodstream of the animals to reach the tumor sites at a therapeutically effective concentration, as well as the effectiveness of δ-T against human derived tumors *in vivo*. This data supports the viability of δ-T as a chemopreventive agent with bioavailability through the bloodstream in the absence of supra-nutritional α-T supplementation's negative impacts.

The reduction in bladder size and urinary tract weight of the UPII mutant Ha-ras transgenic mouse model further demonstrates the efficacy of dietary δ tocopherol administration at the natural primary bladder tumor sites on the interior epithelium of the bladder. Importantly, observation of mice over the course of the treatment showed no signs of adverse effects and necropsy after the end of treatment revealed no signs of toxicity, an encouraging indicator of safety for potential future development of δ-T for human use.

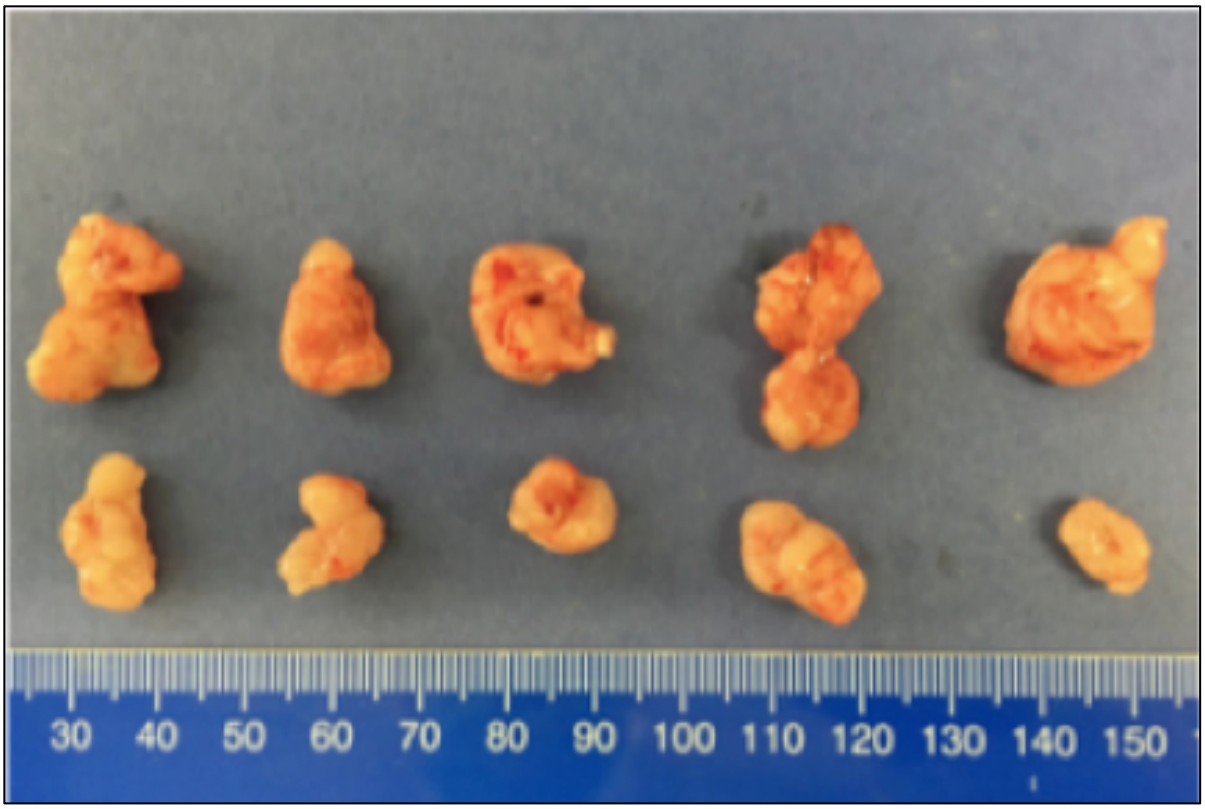

Figure 4.1 Tumors excised from UM-UC-3 Xenograft model

All tumors collected from female nude mice subcutaneously implanted with $1x10^6$ UM-UC-3 MIBC cells and fed for two weeks with either vehicle control (top row) or 0.2% w/v δ–T (bottom row) diet.

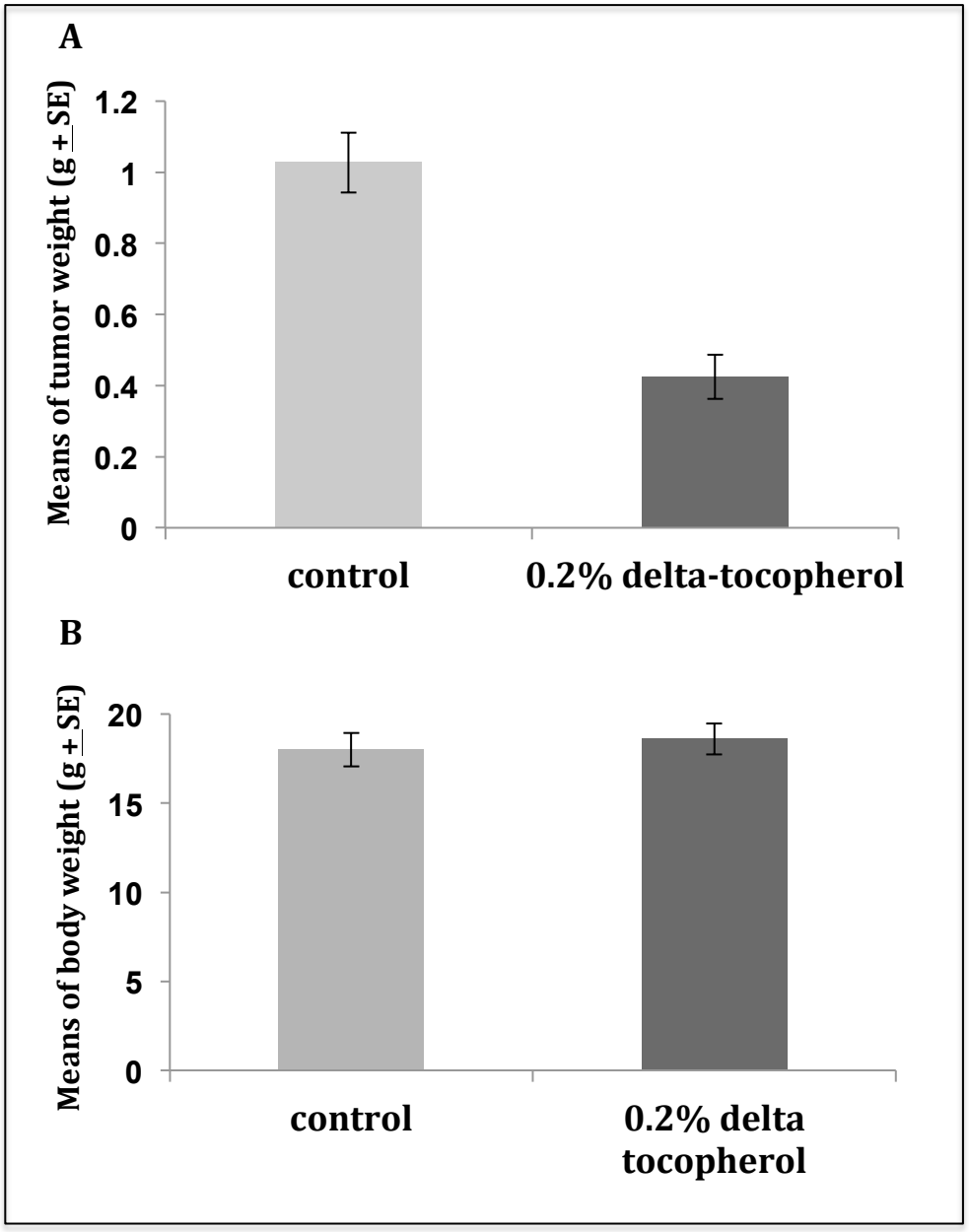

Figure 4.2 Average body weight and tumor weight of UM-UC-3 xenograft model mice

A: Average body weight of mice fed with vehicle control or 0.2% w/v δ–T diet was not significantly different at the experimental endpoint. B: Average tumor mass of δ–T fed mice was 58.6% (p=.000434) lower than tumor mass of control mice.

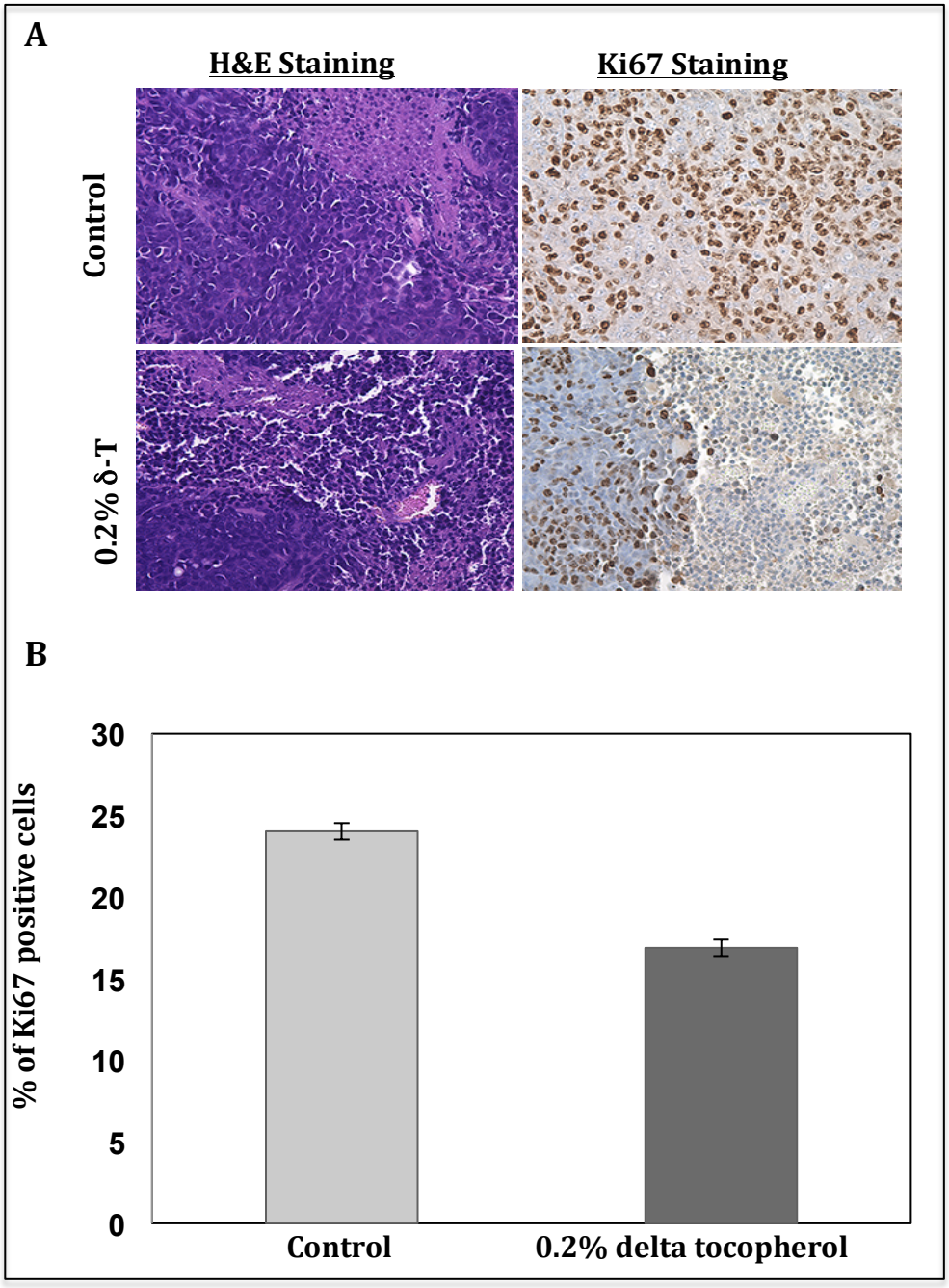

Figure 4.3 Average percentage Ki-67 positive cells in xenograft tumors

A: H&E and Ki-67 stained mouse tumors. B: % Ki-67 positive cells. Treated mouse tumors exhibited 7.14% (p=.0058) fewer Ki-67 positive cells.

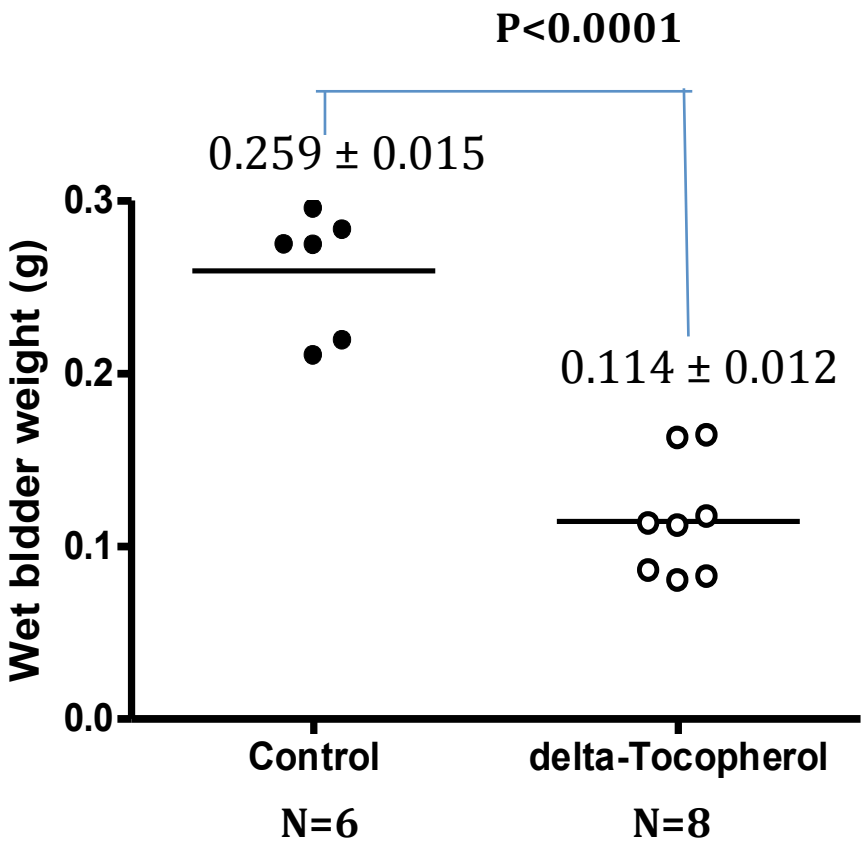

Figure 4.4 Wet bladder weights of UPII Mutant Ha-Ras mice

Genitourinary tracts of survived UPII Mutant Ha-Ras mice were weighed as a surrogate measurement for tumor burden. δ–T fed mice exhibited an average of 55.99% reduction in genitourinary tract weight (p<.0001).

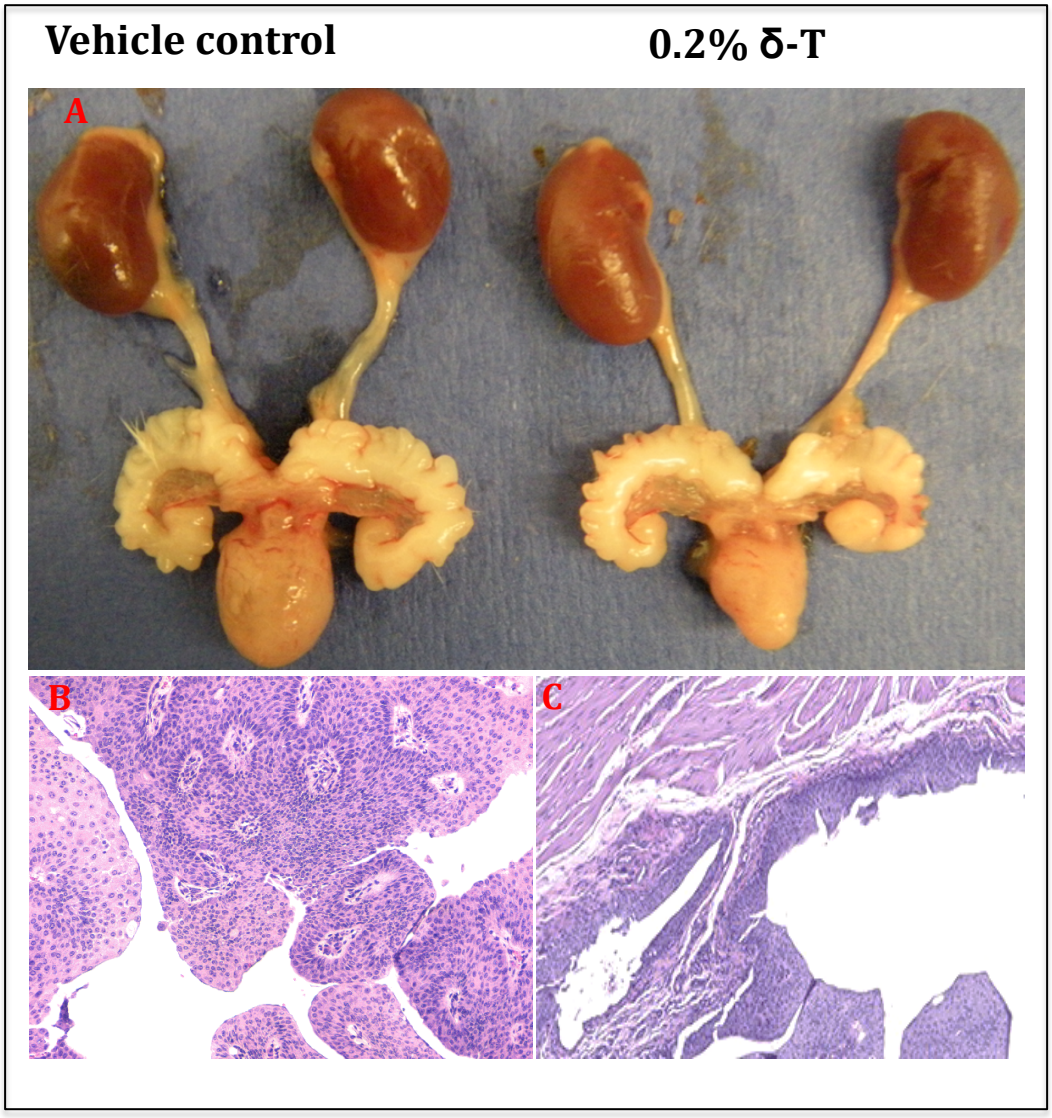

Figure 4.5 Genitourinary tracts of UPII Mutant Ha-Ras mice

A (left) and B: Whole genitourinary tract and H&E stained bladder tissue section from representative vehicle control fed mice. A (right) and C: Whole genitourinary tract and H&E stained bladder tissue section from representative δ–T fed mice.

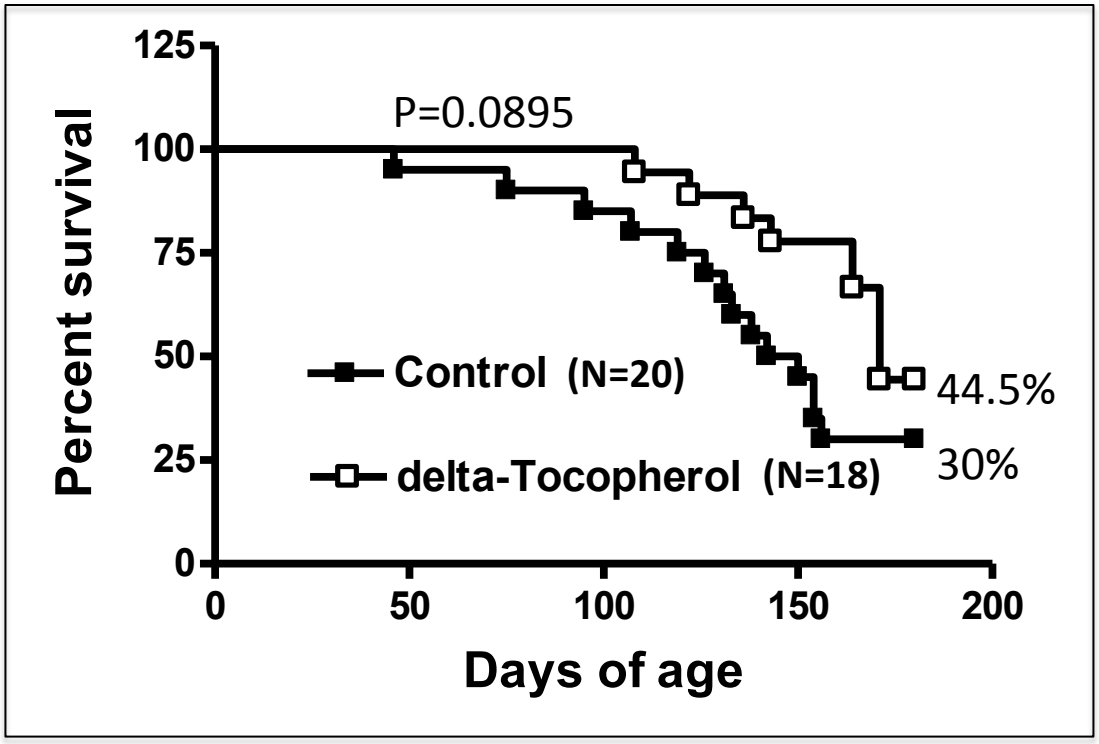

Figure 4.6 Survival curve for UPII Mutant Ha-Ras mice

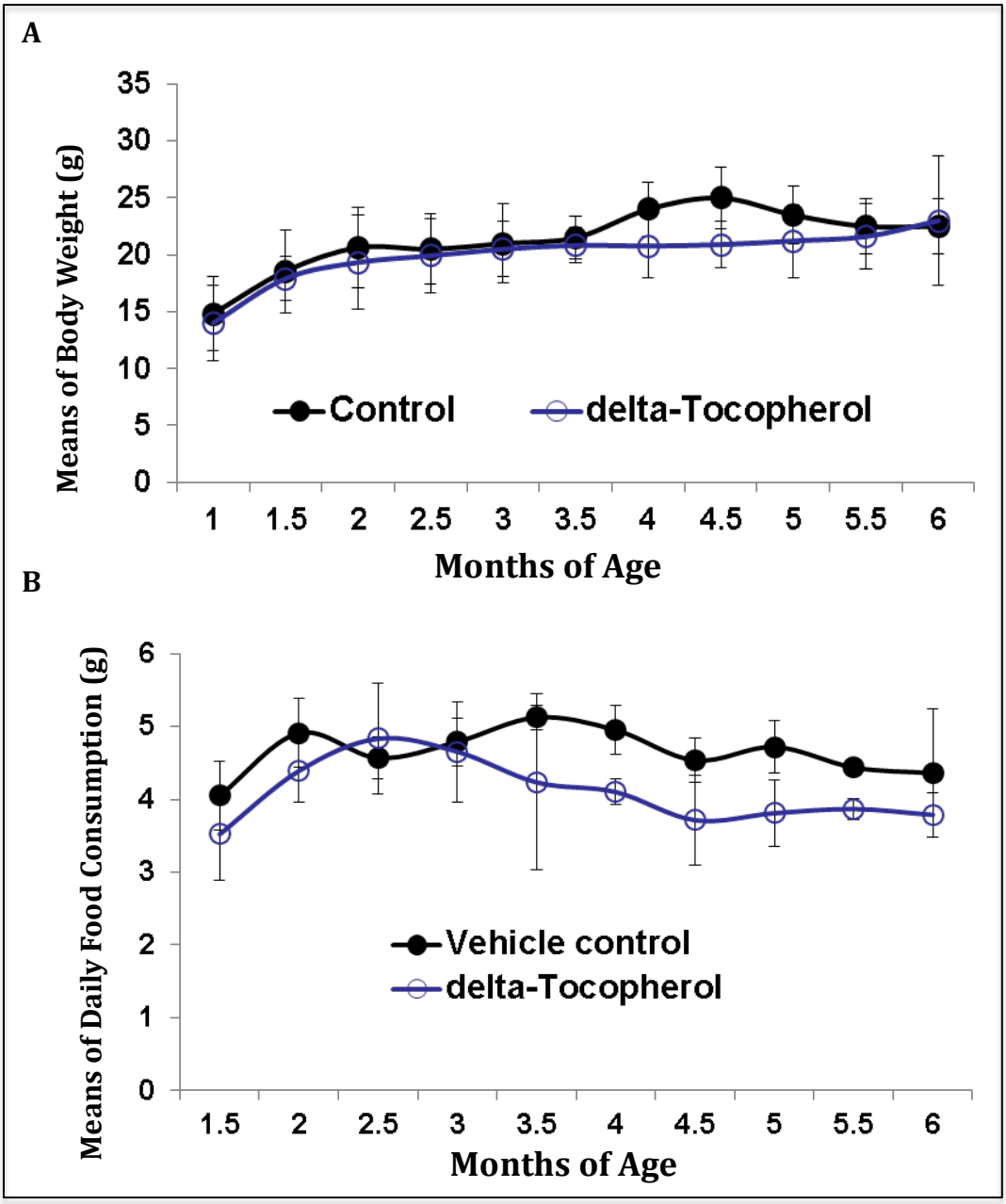

Figure 4.7 Body weight and Daily Food Consumption of UPII Mutant Ha-Ras mice

Chapter 5: Discussion

The development of novel, safe, and inexpensive chemopreventive agents for BCa treatment is a high priority for the improvement of BCa patient quality of life and reduction of the burdens of continuing BCa care. Natural compounds, such as δ-T, which are inexpensive and may be administered by dietary supplementation, are exciting potential agents for development in this context. Previous cancer prevention studies of Vitamin E have focused heavily on α- and to a lesser extent γ- tocopherols, which we demonstrate are dramatically less effective in BCa treatment than the δ-tocopherol, which unfortunately naturally occurs at a much lower rate in common dietary Vitamin E sources. Furthermore, α-tocopherol supplementation reduces serum levels of other forms of tocopherol, which could further reduce the detectable benefits of γ- and δ- tocopherols in mixed Vitamin E supplementation. The study of δ-T as an individual agent, which has not been previously conducted in BCa, and has likewise been only minimally studied in other cancers, is therefore more likely to lead to potential clinically applicable findings.

Flow cytometry analysis in combination with MTT metabolic assays demonstrates that δ tocopherol potently and dose-dependently induces apoptosis in human bladder cancer cell lines, and western blotting analysis of treated cell lysates revealed that DR5 expression is strongly upregulated by δ-T treatment. Downstream of DR5, caspase and PARP cleavage, both strong indicators of apoptosis induction, were significantly increased consistent with flow cytometry results. siRNA knockdown of

DR5 during treatment resulted in reduction of δ-tocopherol induced caspase cleavage, as expected. DR5 upregulation was determined to be caused by the ATF4/CHOP-DR5 pathway, which responds to endoplasmic reticulum stress to induce apoptosis. ER stress sensors PERK and IRE1α, and downstream transcription factor ATF4, were highly upregulated and phosphorylated in response to δ-T treatment, indicating strong induction of ER stress and activation of the UPR by δ-T.

Importantly, an unexpected morphological change was induced in BCa cell lines treated with δ-T that was readily apparent under even low magnification conventional light microscopy. This morphology appeared to most closely match that of autophagy, though the autophagosomes observed seemed larger than those appearing under normal circumstances of autophagy. The suspicion that the observed morphological change was indeed autophagy was confirmed by upregulation of autophagic markers and observable accumulation of GFP-tagged LC3 protein in stably transfected BCa cells treated with δ-T. To better observe the autophagic phenotype, I used TEM on control and δ-T treated cells, which revealed a striking difference in the appearance of the cells and the size, number, and frequency of autophagosomes visible within them. Upon reaching the maximum magnification attainable with the available EM, the contents of these autophagosomes appeared to be membranous structures most closely resembling those identified in yeast cells treated with ER-stress inducing agents (Schuck et al. 2014). This observation came as a surprise, as the specific ER-specific mechanism of autophagic degradation has to our knowledge not been described in cancer or indeed in humans, yet the overall autophagic phenotype of δ-T treated BCa cells and ER-stressed

yeast bore a striking resemblance, as did the contents of the autophagosomes. In order to strengthen the evidence that ER degradation is occurring in the large autophagosomes induced by δ-T treatment, immunofluorescent staining of calnexin, a marker for the ER, was used in T24/LC3-GFP cells. The resulting images show colocalization of ER staining and GFP tagged LC3 aggregates, supporting the TEM observations of ER like structures in the autophagosomes.

In vivo efficacy of dietary δ tocopherol was examined to determine if the *in vitro* findings could potentially be further developed as a novel BCa therapeutic mechanism. One of the potential major hurdles to the development of δ tocopherol as a novel chemopreventive agent is the concern that the bioavailability issues caused by αTTP's higher affinity for α-T would prevent oral administration of δ-T from exhibiting a therapeutic effect. (Brigelius-Flohe and Traber 1999, Traber 2007) While this would not have completely negated the potential usage of δ-T in bladder cancer specifically, where there is potential for its use as an intravesical agent, it would have been a blow to the potential application of δ-T in tumor sites best reached via the bloodstream.

The tumorigenesis model provided by the UPII-mutant Ha-ras model demonstrated that δ tocopherol feeding significantly reduced tumor formation in the urinary tract, and that the biological activity of ingested δ tocopherol or its metabolites is retained at the target site on the interior of the bladder after the compound has been exposed to metabolic processes. The success of dietary δ tocopherol administration in the xenograft model is also encouraging, as it is indicative that sufficient concentrations of δ tocopherol are also present in the bloodstream to reach tumor sites outside of the

genitourinary and digestive tracts, which could allow further application of δ tocopherol for the treatment of metastatic cancers or cancers initiated in different organ systems.

Future Directions

While the data presented here provides strong evidence of the induction of ER stress and activation of UPR-mediated apoptotic cell death as the primary mechanism by which δ-T kills bladder cancer cells, there remain aspects of δ-T's effects to be further explored. Chief among the new questions raised by these findings is the exact extent and mechanism of the observed ER degradation in δ-T induced autophagy. While the evidence provided in this study and the mechanism described in existing literature (Schuck et al. 2014) are in agreement, further experiments are warranted to determine the exact role and type of reticulophagy occurring in δ-T treated cells. Ideally, higher magnification and resolution TEM images of the ER in δ-T treated cells and their autophagosome contents would provide more compelling visual evidence of ER expansion and degradation in response to δ-T induced ER stress. Likewise, higher magnification images of immunofluorescently stained δ-T treated cells would strengthen the evidence for reticulophagy beyond what was possible in this study. Knockout of Atg genes and subsequent δ-T treatment and EM imaging would provide further support for a shared mechanism with yeast cells of ER specific degradation independent of normal autophagic machinery. An additional potential mechanism of cell death with a visually similar phenotype to the one observed in this study has

recently been described and named “autosis” (Liu et al. 2013). Though this mechanism is not yet fully defined and accepted into the cell death lexicon, and its linkage to the UPR is less clearly defined than the mechanism described in yeast (Schuck et al. 2014), it is linked to cell death caused by extreme autophagy induction that ultimately depletes both the ER and nuclear membranes in an Atg dependent and Na^+,K^+ATPase mediated fashion, and may be an additional potential mechanism for future examination.

Future studies on δ-T would also benefit from a larger population size and follow-up time of UPII mutant Ha-Ras transgenic mice and different δ-T dose levels, in order to establish a statistically significant result with regards to overall survival benefit of δ-T supplementation. There are additionally multiple potential agents for evaluation as therapeutics in combination with δ-T supplementation, including clinically approved proteasomal inhibitors such as Bortezomib (Adams and Kauffmann, 2004), Carfilzomib (Vij, 2012), Nelfinavir (Gills et. al., 2007), and Marizonib (Chauhan et. al., 2006), as well as various other agents targeting individual components of the cell’s protein folding machinery or the UPR such as chaperone inhibitors DHA (Begum et. al., 2014) and PAT-SM6 (Hensel et. al., 2013) or UPR inhibitors 6-shogaol (Hu et. al., 2012), GSK2656157 (Axten et. al., 2013), GSK2606414 (Axten et. al., 2012), STF-083010 (Papandreou, 2011), and MKC-3949 (Mimura et. al., 2012). For BCa specifically, δ-T could also feasibly be developed into an intravesical therapeutic agent, though this more invasive use of the compound would more likely be applied as an adjuvant treatment than as a preventative measure as dietary δ-T could be used.

Conclusion

As an exceptionally burdensome disease on both patients and the healthcare system as a whole, BCa is an ideal candidate for the development of novel agents that may be used in a non-invasive, dietary intervention to prevent recurrence or reduce its recurrence rate. Natural compounds already present in the diet, such as the tocopherols found in Vitamin E, can provide attractive and safe candidates for development as these types of agent. As past clinical studies showed limited or mixed effectiveness of dietary Vitamin E supplementation, I sought to evaluate the potential of tocopherols as individual agents and determined that the less dietarily prevalent δ-T was the most effective candidate for further study.

δ-T potently induced apoptosis in cell lines of both NMIBC and MIBC, demonstrating its potential for clinical application against both early stage and the more dangerous metastatic BCa and favorable safety characteristics in non-cancerous immortalized urothelial cells. This study demonstrates that the apoptotic cell death induced by δ-T treatment of BCa occurred via a DR5 mediated mechanism induced by ER stress and UPR hyperactivation, and could be attenuated by CHOP or DR5 knockdown. A potential role for autophagy in the cell death process induced by δ-T treatment was also identified, adding a highly novel and potentially clinically relevant finding to the limited existing data on the role of autophagy in resolving ER stress situations.

The safety and effectiveness of dietary δ-T supplementation *in vivo* was clearly demonstrated in two mouse models of BCa, offering encouraging prospects further pursuit of δ-T supplementation in human clinical trials. Both long and short-term feeding with δ-T caused no observable harmful effects in mice, while dramatically reducing the tumor burden in both treated mouse models. These mouse studies provided important evidence that a dietary route was sufficient for δ-T treatment to effectively impact tumor growth and tumorigenesis and that the pharmacokinetic difficulties of dietarily ingested δ-T could be overcome.

In summary, this study highlights the potential for the development of δ-T as a novel, safe, non-invasive, and low-cost agent for the treatment of BCa. With its favorable safety characteristics as a component of a dietary vitamin and its ER-stress based mechanism of action with broad applicability to other cancer types and disease states, δ-T has the potential to overcome the problems which plagued clinical studies of Vitamin E supplementation and become an effective means of improving the treatment outcomes and lives of cancer patients around the world.

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