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Investigation of the effects of α-TEA, 9-Nitrocamptothecin and Paclitaxel Alone and in combination on 66cl-4-GFP Murine Mammary Cancer Cells *in Vitro* and *in Vivo*

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by

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Dedication

To my parents for always being there and for giving me unconditional love and support. To my friends who make me laugh and pick me up when I am down.

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Investigation of the effects of α-TEA, 9-Nitrocamptothecin and Paclitaxel Alone and in combination on 66cl-4-GFP Murine Mammary Cancer Cells *in Vitro* and *in Vivo*

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Second only to lung cancer, breast is the leading type of cancer among women in the US. Despite all the medical advances over the past few decades, toxicity and increased resistance to standard drug therapy still remains a significant problem. The heterogeneic nature of all cancers has led to a shift in treatment approaches, in that more research is being carried out with combination treatments in the hope that a multidirectional targeting of cancer will be far more effective than the current single treatment options. Our goal was to gain a better understanding of the molecular mechanisms of a non-hydrolyzable ether analog of RRR- α -tocopherol, 2, 5, 7, 8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid (abbreviated α -TEA), and to investigate its

efficacy when used in combination with known chemotherapeutics 9-nitro-camptothecin (9NC), and Paclitaxel (Taxol). The data presented here looks encouraging as it shows a clinically relevant delivery method using α-TEA and 9NC has the unique ability to reduce primary tumor burden as well as macro and micrometastatic lung and lymph node lesions in an aggressive syngeneic mouse mammary model, while displaying no obvious toxic side effects. The effect of combination treatments on tumor volume appears in part to be moderated by an increase in tumor cell apoptosis and a decrease in tumor cellular proliferation. Next, the intricate molecular mechanism of how α-TEA alone and in combination with 9NC is able to induce apoptosis in 66cl-4-GFP murine mammary cancer was investigated. The data suggest that the signaling pathway that ultimately leads to apoptosis is caspase dependent, is able to upregulate pro-death players while at the same time downregulate pro-survival proteins such as c-Flip and survivin. Finally, we investigated the efficacy of α-TEA used in an allograft mouse model following treatment with Taxol. Combination treatments were able to significantly reduce primary tumor burden, decrease lung and lymph node micrometastases, tumor cell proliferation, tumor blood vessel density as well as increase tumor cell apoptosis. Based on the results presented, we propose that α-TEA when used alone and in combination is an effective, non-toxic option for cancer treatment which warrants further investigation.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Cancer Incidence and Background

The beginnings of the modern era of cancer chemotherapy research can be traced back as far as the 1940's. In the last 60 plus years there have been giant leaps from developmental therapeutics and early clinical trials to community-wide applications, in the hope of gaining a better understanding of cancer causes, progression and possible treatments. In recent years, even though there has been an explosion of life-saving treatment advances against cancer, the rates of cancer incidence and cure rates have remained relatively unchanged in comparison to other leading causes of human mortality such as cardiovascular disease.

Breast Cancer is the most common type of cancer diagnosed among women in the U.S. In 2007, roughly 42 million mammograms will be performed, of which an estimated 178,480 women will ultimately be diagnosed with invasive breast cancer (1). Breast Cancer rates continue to climb, with more than 1.2 million breast biopsies performed per year (1). An estimated 40,460 women will die of breast cancer in 2007, making breast cancer the second leading cause of cancer deaths in women, after lung cancer (1). It is estimated that in 2007, breast cancer will have the highest reported incidence of new cases (26%) when compared to all other forms of cancer and will account for approximately 15 percent of all cancer deaths in females (1). With this in mind, the need for novel and effective treatments against breast cancer becomes of the utmost importance.

Previously there were only one or two options for treating cancer, today there's an overwhelming menu of treatment choices that target the complex mix of cells in each individual cancer. The decisions are surgery, hormonal (anti-estrogen) radiation therapy and/or chemotherapy. The latter two options are preferred in most cases following surgery (1).

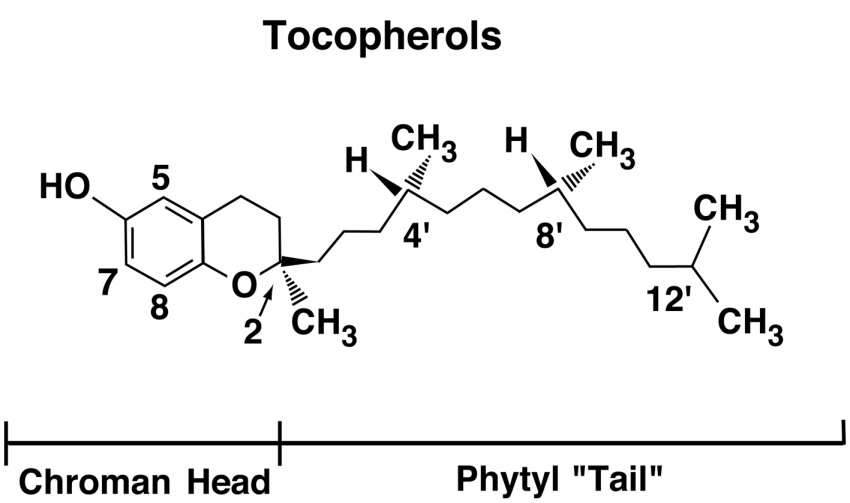
Scientists have been discussing what is the best method for tackling cancer for years, but it is universally agreed that there are two main arenas in which this problem can potentially be addressed; genetic and epigenetic factors. Since genetics are next to impossible to alter, the main focus of prevention and treatment have rested on epigenetics such as diet and environment, since they are inherently easier to change. Taking this into account, the scientific community has paid particular attention to compounds that are naturally occurring and biologically active; they are either found in the environment or in the human diet. One of their main attractive properties is that they exhibit low toxicity even at high doses. The uses of such compounds appear to becoming full circle as they have been playing pivotal roles in natural medicine for many years.

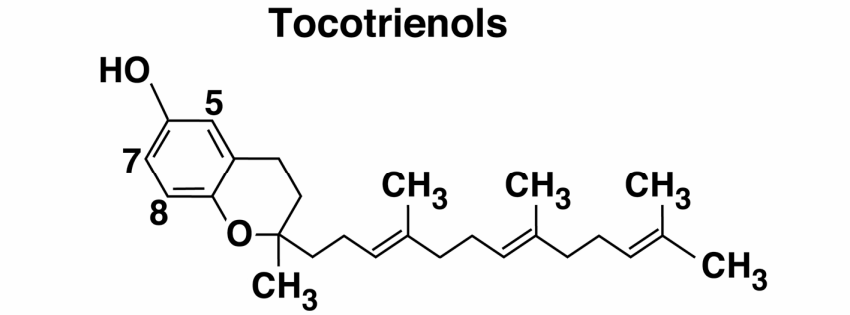
Talking this into account, the studies in our lab have focused on Vitamin E and several of its derivatives, namely α-TEA, as they exhibit low toxicity and have been shown to affect many pro-apoptotic pathways (2,3,4,5,6,7). It is proposed that α-TEA when used in conjunction with known chemotherapeutics, 9NC and Paclitaxel will be able to decrease the threshold of cell killing, providing greater efficacy and fewer adverse side affects.

Vitamin E

History

Vitamin E was first identified in 1922 by Evans and Bishop as a dietary factor after observing that rats fed a lipid deficient diet showed increased infertility (8). This factor was later isolated, characterized and called vitamin E (8). Today the term vitamin E refers to a group of eight naturally-occurring lipid soluble compounds, as well as synthetic vitamin E. These structurally related compounds contain both a chromanol head and a phytyl tail; the differences between them lie in the placement of the methyl groups on the chroman head, as well as the presence or absence of double bonds on the phytyl tail (9,10). The eight compounds are divided into tocopherols (α,β,γ,δ), that possess a saturated phytyl tail and tocotrienols (α,β,γ,δ) that have an unsaturated phytyl tail (Fig 1.1). Vitamin E compounds are further classified by the stereochemical arrangement of the methyl groups found at the 2 position of the ring structure as well as the 4' and 8' positions on the phytyl tail. The R designation refers to the right hand position and the S designation to the left hand position. Vitamin E in its natural forms have these carbons in the R position, thus are known as RRR tocopherols (9,10). α-tocopherol has been shown to be the most abundant natural form and exhibits the highest biological activity of the vitamin E compounds (Table1.1) (9,10).





Position of methyls	Tocopherols	Tocotrienols
5,7,8	α-tocopherol	α-tocotrienol
5,8	β-tocopherol	β-tocotrienol
7,8	γ-tocopherol	γ-tocotrienol
8	δ-tocopherol	δ-tocotrienol

Figure 1.1: Structure of natural vitamin E compounds; tocopherols and tocotrienols. The eight different stereoisomers have shown different activity levels *in vivo*.

Table 1.1: Relative Biological Activity (based on rat fetal resorption studies) of Natural Tocopherols and Synthetic Sterioisomers. (9,10)

	% Activity
Natural derivatives	
RRR-α-tocopherol	100
RRR-β-tocopherol	57
RRR-γ-tocopherol	37
RRR-δ-tocopherol	1.4
8 Stereoisomers – Synthetic Vitamin E	
RRR-α-tocopherol	100
RRS-α-tocopherol	90
RSS-α-tocopherol	73
SSS-α-tocopherol	60
RSR-α-tocopherol	57
SRS-α-tocopherol	37
SRR-α-tocopherol	31
SSR-α-tocopherol	21

Known Functions

Vitamin E has historically been characterized as a lipid soluble antioxidant. Along with other micronutrients and enzymes, vitamin E provides defense against damage induced by harmful oxygen products. Oxygen used for energy production leads to the creation of free radicals, which in turn have the ability to react with unsaturated bonds that are present in lipid membranes that cause denaturing of proteins and are detrimental to nucleic acids. Cellular damage of this nature has been associated with various age related disorders such as atherosclerosis and cancer (11). Vitamin E has been shown to have the ability of scavenging these harmful free radicals and quenches them via termination of

lipid peroxidation. It has also been reported that vitamin E may play a role in membrane stabilization, cell signaling and the inhibition of cell proliferation (12,13). These characteristics of vitamin E make it an obvious choice to study its effects on various types of cancer.

Vitamin E Compounds – Anticancer effects and Investigation of Molecular Mechanisms

Absorption

As previously mentioned vitamin E is a lipid soluble molecule and is absorbed by the body with ingested lipids (11). The formation of chylomicrons occurs in the gastrointestinal tracts, promoting absorption of fat soluble vitamins, cholesterol, apolipoproteins and phospholipids. Chylomicron remnants are then formed by the action of lipoprotein lipase on the chylomicrons that have been transported to liver via the lymphatic system. These remnants and the associated vitamin E are recognized and taken up by the apolipoprotein-E receptors located on the parenchymal cells of the liver into the hepatocytes. In the hepatocytes there is a repackaging of the chylomicron remnants into very low density lipoproteins (VLDL's). Considering that vitamin E is hydrophobic in nature, there is a necessity for a binding protein that aids in the transport of vitamin E into and around the cell. This binding protein is named α-tocopherol transfer protein (α-TTP) (14). α-TTP protein has been shown to be exclusively expressed in the liver and

mediates the transfer of α-tocopherol between membranes. α -TTP preferentially incorporates α-tocopherol into VLDL's, in the process leaving the majority of the other forms to be excreted with bile (14). It is α-TTP that enables the liver to discriminate between the various forms of vitamin E and α-TTP's affinity for the various forms correlate with their biological availability (15,16). After packaging into VLDL's, vitamin E can then be secreted by the liver cells into the blood (15). Absorption of vitamin E by peripheral cells involves high density lipoproteins (HDL's) or metabolized VLDL's, known as low density lipoproteins (LDL's) (15).

Storage, Metabolism and Excretion

Vitamin E has been found in high amounts in adipose tissue as it is a fat soluble vitamin that requires a non-aqueous environment. As previously stated vitamin E is present in liver cells as well as in nerve cells, the pancreas and adrenal gland (16). Normal human plasma levels of vitamin E are typically in the range of 20-30μM (9). The metabolism of vitamin E itself is not fully clear. The antioxidant role that vitamin E has historically played has prompted the investigation of oxidized metabolites of the vitamin. Quinone and hydroquinone forms of the vitamin have been found in membranes after vitamin E's interaction with peroxyl radicals (9, 17). Another proposed metabolite of vitamin E has been identified in the urine and is now known as α-CEHC (2, 5, 7, 8-tetramethly-2(2-carboxyethyl)-6-hydroxychroman). This metabolite appears to be due to the clipping of the phytyl tail by two carbons while leaving the chroman head unchanged (17).

Sources and Availability

Primary sources of vitamin E in the diet are whole grains. Other sources include seeds, oils such as palm, soybean, sunflower, safflower and corn oil as well as being found in nuts. Vitamin E can be purchased not only in the natural (RRR- α-tocopherol) form but also in a synthetic (all-rac-α-tocopherol) form. This synthetic form is a mixture of the eight stereoisomers created by the chiral centers located on carbon 2 of the chroman head and carbons 4' and 8' on the phytyl tail (Fig 1.2). It should be noted that these synthetic forms of vitamin E are less biologically active than the naturally occurring form based on rat resorption bioassay (9).

Deficiency and Toxicity.

Vitamin E deficiency has been observed in individuals with severe malnutrition, genetic defects affecting the alpha-tocopherol transfer protein, and fat malabsorption syndromes. For example, children with cystic fibrosis or cholestatic liver disease, who have an impaired capacity to absorb dietary fat and therefore fat-soluble vitamins, may develop symptomatic vitamin E deficiency. Severe vitamin E deficiency results mainly in neurological symptoms, including impaired balance and coordination (ataxia), injury to the sensory nerves (peripheral neuropathy), muscle weakness (myopathy), and damage to the retina of the eye (pigmented retinopathy). For this reason, people who develop peripheral neuropathy, ataxia or retinitis pigmentosa should be screened for vitamin E deficiency (18). The developing nervous system appears to be especially vulnerable to vitamin E deficiency because children with severe vitamin E deficiency from birth, who

are not treated with vitamin E, develop neurological symptoms rapidly. In contrast, individuals who develop malabsorption of vitamin E in adulthood may not develop neurological symptoms for 10-20 years. It should be noted that symptomatic vitamin E deficiency in healthy individuals who consume diets low in vitamin E has never been reported (19).

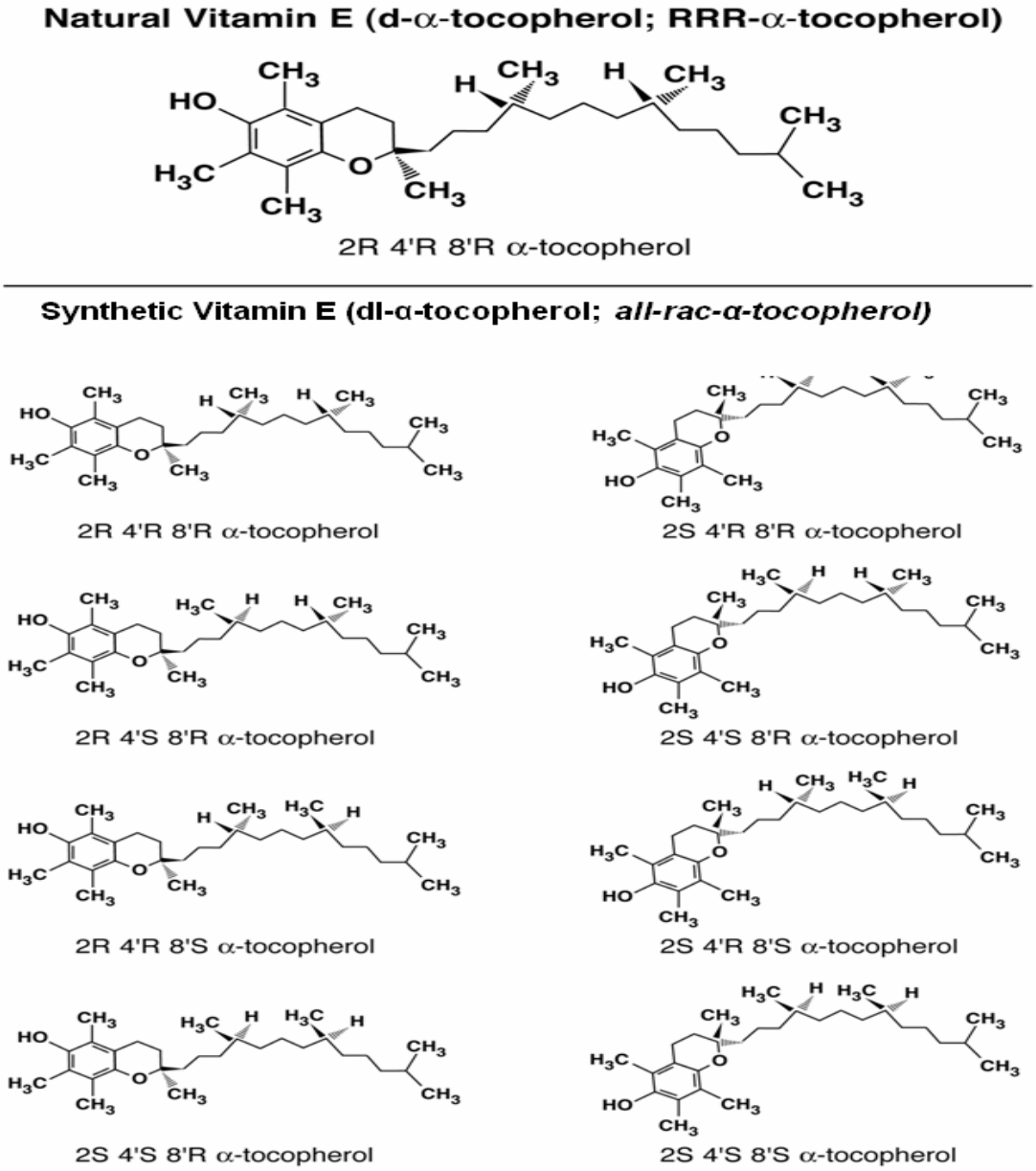


Figure 1.2: Natural and Synthetic forms of Vitamin E (9).

RDAs for vitamin E are based only on the alpha-tocopherol form of vitamin E. According to the U.S. Department of Agriculture (USDA), the intake of vitamin E by women 19–50 years of age averages less than 90 percent of the RDA. Americans

consume roughly 7–9 milligrams compared to the recommended 15 milligrams. Vitamin E deficiency is rare in humans. Three specific situations are likely to cause vitamin E deficiency. People who cannot absorb dietary fat due to an inability to secrete bile or with rare disorders of fat metabolism are at risk of vitamin E deficiency (20). Individuals with rare genetic abnormalities in the alpha-tocopherol transfer protein are at risk of vitamin E deficiency (21) and premature, very low birth weight infants (birth weights less than 1500 grams, or 3 pounds, 4 ounces) are at risk of vitamin E deficiency (22)

Blood levels of vitamin E may also be decreased with zinc deficiency (23). Vitamin E deficiency is usually characterized by neurological problems associated with nerve degeneration (17).

Vitamin E Derivatives

α –TEA + VES

Until the synthesis of α-TEA, RRR-α-tocopherol succinate (VES, vitamin E succinate) was considered to be the most effective form of vitamin E capable of inhibiting tumor cell growth (Fig 1.3). Studies suggested that the tumor inhibitory functions of VES were due to the intact form of VES being maintained, as cleavage of the succinic acid moiety from RRR-α-tocopherol succinate, led to a loss in its ability to exert its anti-cancer abilities (25). As a direct result of these findings there was a need for a novel form containing a non-hydrolyzable ether linkage attaching an acetic acid moiety to RRR-α-tocopherol (Fig. 1.3).

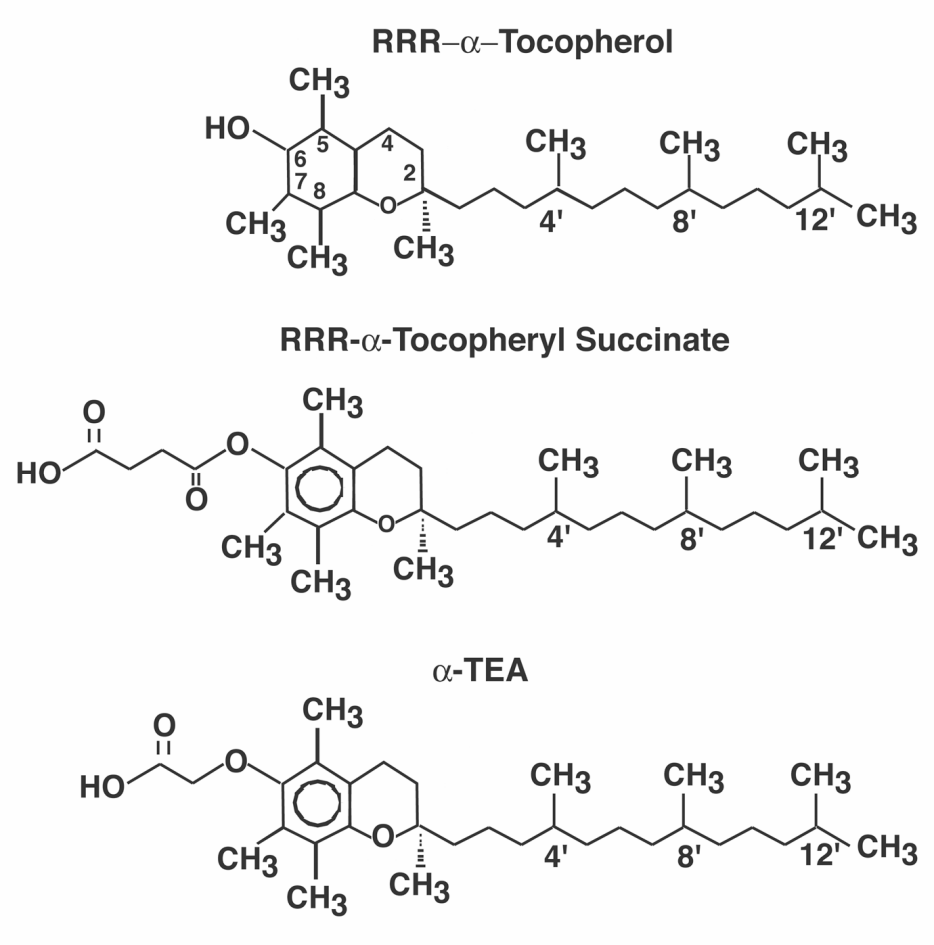


Figure 1.3: Compares natural vitamin E (RRR-α-tocopherol) with vitamin E derivatives RRR-α-tocopherol succinate (VES, vitamin E succinate) and 2, 5, 7, 8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA). VES consists of RRR-α-tocopherol attached with a succinic acid moiety at carbon 6 of the chroman head via an ester linkage. α-TEA contains an acetic acid moiety instead of the hydroxyl group at carbon 6 attached via a non-hydrolyzable ether linkage.

The non-hydrolyzable ether analog of RRR- α -tocopherol, 2, 5, 7, 8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid (abbreviated α -TEA) was

developed to meet this need. Previous studies have shown that α -TEA has an intrinsic ability to induce apoptosis in a concentration- and time-dependent manner of murine mammary 66cl-4-GFP tumor cells *in vitro*. It has also been shown to significantly decrease primary tumor burden as well as reduce lung and lymph node micrometastasis in Balb/c mice transplanted with 66cl-4-GFP cells (26). The incorporation of α -TEA into a liposomal aerosol treatment fulfills the desire to make a non-toxic, effective vitamin E-based chemotherapeutic agent that is not only clinically useful but can be delivered in a clinically applicable Fashion (26). Previous studies in our lab have highlighted the potent ability of α -TEA to induce apoptosis in a wide variety of tumor cells lines namely, human breast (MCF-7, MDA-MB-231, MDA-MB-435), ovarian (A2780, A2780/cp-70), cervical (ME-180), endometrial (RL-952), prostate (LnCaP, PC-3, DU-145), colon (HT-29, DLD-1), lung (A-549) cells while having no adverse effects on normal human mammary epithelial cells (HMEC) or immortalized non-tumorigenic MCF-10A human mammary epithelial cells. (25). There is a need for new strategies in the treatment of cancer that allows for effective cancer cell death without the common side effects that accompany high dose therapies. Vitamin E and several of its derivatives, namely α-TEA, are suitable choices as they exhibit low toxicity and high effectiveness *in vivo* and have been shown to affect many pro-apoptotic pathways (27).

Programmed Cell Death - Apoptosis

For every cell, there are two ultimate roads that it may go down. One that leads to its survival and one that eventually results in its demise. The concept of cell death was first

reported in 1842 by Carl Vogt when studying the tadpole of the midwife toad (28), and from then until now the subject of programmed cell death (apoptosis) has been one of the most studied fields in the scientific community. Cell death plays one of the most important roles in biological systems, so the question begs to be asked; why should a cell commit suicide? There appear to be several different reasons. Programmed cell death is as needed for proper development as mitosis is. The formation of the fingers and toes of the fetus requires the removal, by apoptosis, of the tissue between them. The sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation occurs by apoptosis. Secondly, programmed cell death is needed to destroy cells that represent a threat to the integrity of the organism, such as cells with DNA damage due to radiation or spontaneous mutations. One of the methods by which cytotoxic T lymphocytes (CTLs) kill virus-infected cells is by inducing apoptosis. As a direct result of cell-mediated immune responses, the effector cells must be removed to prevent them from attacking body constituents. CTLs induce apoptosis in each other and even in themselves. The effect of an inability to perform this function is highlighted in autoimmune diseases such as lupus erythematosus and rheumatoid arthritis.

Characteristics of cells that are undergoing apoptosis include a visible shrinkage of the affected cell. This is then followed by DNA fragmentation, condensation of the cell's chromatin and then eventual blebbing of nuclear and cytosolic products. (28,) These apoptotic bodies are then removed by phagocytic cells via endocytosis. (29,30,31,32,33, Fig 1.4)

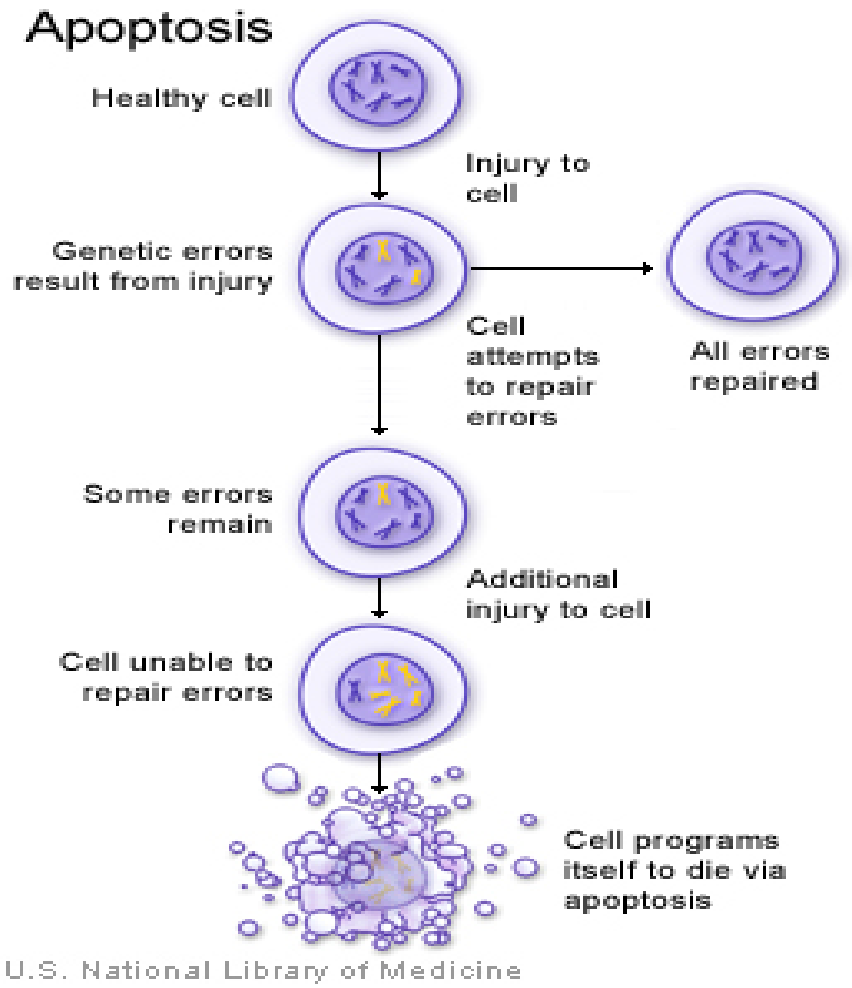


Fig 1.4: Apoptosis - the programmed death of a cell

As previously mentioned the reasons why cells undergo apoptosis cover a wide spectrum. How they initiate their own demise is understandably a complex story too. These death signals are either initiated internally (intrinsic or mitochondrial pathway) or externally (extrinsic). Within both of these pathways there are a myriad of intermediate players, each one performing a specific role helping either to the survival or eventual death of the cell. These proteins can be grouped into either pro-apoptotic or anti-apoptotic families.

This molecular complexity lends itself in numerous directions in which to tackle apoptosis and its role in cancer treatment, making research in this area very exciting.

The extrinsic or death receptor pathway begins with the binding of a extracellular ligand to a membrane bound death receptor. (30, 32,34). Death Receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily. Members of that TNFR family are diverse in primary structure but all of them consist of cystein rich extracellular subdomains that are thought to adopt generally similar tertiary folds. There are 6 known death receptors; these include Fas (CD95, Apo1), TNFR1 (p55, CD120a), DR3 (Apo3, WSL-1, TRAMP, LARD), DR4 (TRAIL-R1), DR5 (Apo2, TRAIL-R2, TRICK2, KILLER) and DR6. (35) It is the unique structural features of individual family members that allow them to recognize their ligands, CD95L (FasL), TNF and Lymphotoxin alpha, Apo3L (TWEAK) and TRAIL (Apo2L), (27,31,34,35) with specificity and, in most cases, exclusivity (36). Once the receptor has been bound by its ligand, homotrimerization of the receptor occurs, in the process causing the formation of a homologous cytoplasmic sequence termed the "death domain". This is followed by recruitment of death containing domains in adapter-molecules such as FADD, TRADD or DAXX. This allows them to interact with the death receptors and transmit the apoptotic signal to the next player in the death machinery.

Caspases and Apoptosis

The caspases are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within

the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis.

Formation of the DISC typically results in the activation of an initiator caspase such as caspase 8 or caspase 10. At this stage the decision to enter mitochondrial independent (Type I) apoptosis vs. mitochondrial dependent (Type II) is made. Following Type I apoptosis these caspases eventually leads to the activation of the effector caspases, such as caspase 3. Mitochondrial dependent apoptosis involves the activation of key pro-apoptotic and inhibition of anti-apoptotic players. Activated Caspase-8 goes on to truncate Bid (tBid) which acts to inhibit a pivotal anti-apoptotic player Bcl-2. This in turn allows Bax to initiate mitochondrial permeability transition (MPT). This in essence causes pores to be formed in the membrane of the mitochondria allowing cytochrome c to migrate to the cytoplasm. (28,32,34,35). Cytochrome c in association with apaf-1 and pro-caspase-9 form the apoptosome (28, 29,32,34,35). Finally Caspase-9 is activated via the apoptosome causing subsequent Caspase-3 activation and eventual apoptotic cell death (28, 29,32,34,35).

Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway occurs when a healthy cell, suffers internal damage (e.g., from reactive oxygen species). This causes Bid present on the mitochondrial membrane to associate with a related protein, Bax, which punches holes in the outer mitochondrial membrane, causing cytochrome-c to leak out and follow the same sequence of events previously mentioned.

Many tumors overexpress members of the inhibitor of apoptosis protein (IAP) family, such as cIAP1, cIAP2, XIAP (X-linked inhibitor of apoptosis) and survivin, as well as anti-apoptotic proteins Bcl-2 and c-Flip(L). On one hand IAPs contribute to tumor cell apoptosis resistance by the inhibition of effector caspases such as caspases 9 and 3, but also act as a target for specific inhibitor of IAPs, such as the apoptosis inducing factors (AIF), Smac/DIABLO. (28,34). Recent studies have showing inhibiton of anti-apoptotic players such as c-Flip(L) by 9NC and α-TEA and survivin by α-TEA (37) suggest that this is a relevant scientific direction to pursue.

Transforming Growth Factor Beta (TGF-β)

TGF-β is a multifunctional protein which comes in five isoforms, known as TGF-beta-1 , TGF-beta-2 , TGF-beta-3 , TGF-beta-4 , TGF-beta-5, and they are members of the transforming growth factor beta superfamily. The roles that TGF-β play are vast and only act to highlight its importance in cellular regulation. These roles include, differentiation, proliferation and programmed cell death (44,45,46).

The Structure of TGF-β

The peptide structures of the three members of the TGF-β family share high levels of homology. They consist of an N-terminal signal peptide of 20-30 amino acids that is required for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a 112-114 amino acid C-terminal region that becomes the *mature* TGF-β molecule following its release from the pro-region by proteolytic cleavage. The mature

TGF-β protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs (44,45,46,47).

TGF-β has nine conserved cysteine residues, but it is the regions between the fifth and sixth cysteines that exhibit the most diversity within the TGF-β members. Because of this diversity, it is not unexpected that this is the area associated with receptor binding and specificity of TGF-β. They are all encoded as large protein precursors; TGF-β1 contains 390 amino acids and TGF-β2 and TGF-β3 each contain 412 amino acids. They each have an N-terminal signal peptide of 20-30 amino acids that they require for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a 112-114 amino acid C-terminal region that becomes the mature TGF-β molecule following its release from the pro-region by proteolytic cleavage (44).

Functions of TFG-β

Role in apoptosis

Along with death receptors like Fas and TRAIL, TGF-β can also induce apoptosis via either the SMAD pathway or DAXX pathway (51,52,53,54). SMAD and DAXX pathways are the most associated with TGF-β. The mechanism of action follows a more classical sequence of events where TGF beta dimers binds to a type II receptor which in turn recruits and causes phosphorylation of the type I receptor. Downstream R-SMAD is phosphorylated by the type I receptor, which then allows association with SMAD4 forming a heteromeric complex. Apoptosis is eventually triggered when the R-SMAD/SMAD4 complex is able to enter the nucleus where it activates the mitogen

activated protein kinase (MAP kinase) pathway. Another path that TGF-β can follow to induce apoptosis is via association with death associated protein 6 (DAXX adapter protein (51,52,53,54).

9-Nitrocamptothecin/(9NC)

It is proposed that α-TEA when used in conjunction with a known chemotherapeutic, 9NC, will be able to decrease the threshold of cell killing, providing greater capability with fewer adverse side affects.

Camptothecin, a naturally occurring anti-cancer agent, was first extracted from the Chinese tree *Camptotheca acuminata* in the early 1960s (38, Fig 1.5). Since this time, several less toxic, clinically active derivatives have been synthesized including CPT (Irinotecan or CPT-11), 9-amino-camptothecin (9AC) and 9-nitro-camptothecin (9-NC or rubitecan) (38). Camptothecin derivatives are used clinically to treat several forms of cancer including breast, ovarian, lung, and colorectal (39). The anti-cancer effects of 9NC are mediated, in part, by blockage of topoisomerase I, induction of apoptosis, and inhibition of DNA synthesis (40,41,42). Normally, topoisomerase I introduces a nick in the DNA backbone allowing the rotation of one strand around the other. This releases the torsional strain which otherwise accumulates in front of the advancing replication fork. The DNA break is extremely transient and is relegated almost immediately at the same time that the topoisomerase I releases the other strand. In the presence of 9NC, the nick is stabilized which is equivalent to a single-strand DNA break. Collision with a replication fork during DNA replication then leads to the formation of a potentially lethal double-

stranded break. This results in the degradation of DNA and death via apoptosis (Fig 1.6)(40).

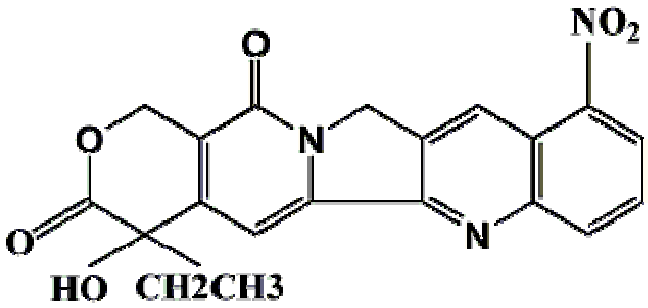


Figure 1. 5: The structure of 9-nitro-camptothecin (9NC) (38).

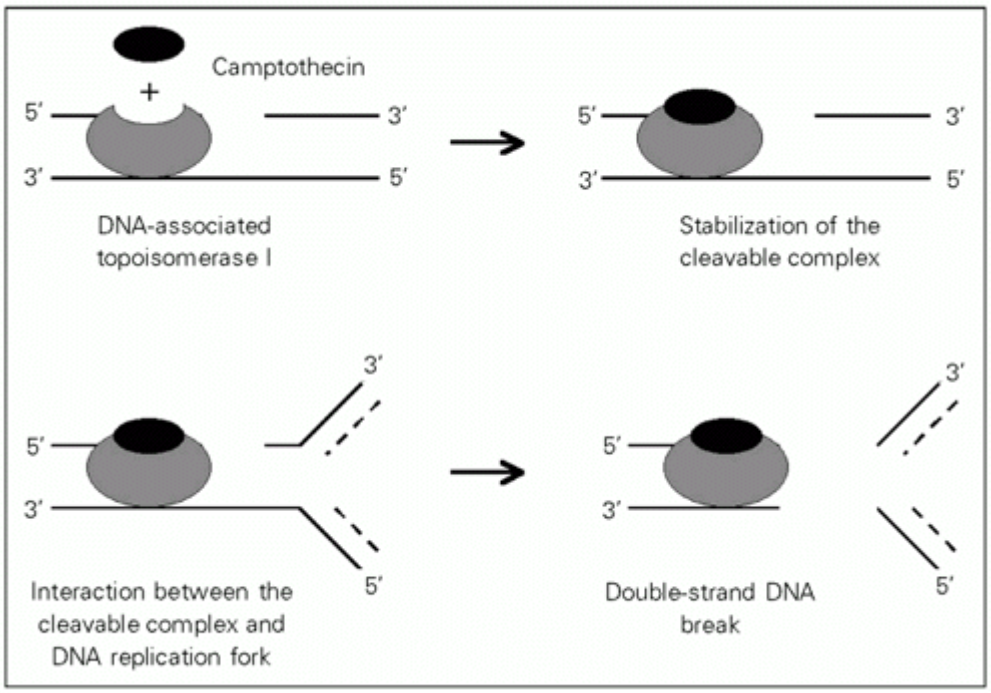


Figure 1.6: Like camptothecin, 9NC acts by introducing single-strand DNA breaks upon the stabilization of DNA-topoisomerase I complexes. As shown these are formed in duplicating DNA as intermediates of topoisomerase I-catalyzed DNA cleavage and religation reactions, necessary to relieve the torsional strain generated by advancing replication forks (131)

Paclitaxel (Taxol)

Paclitaxel is one of the most successful drugs used in the treatment of cancer in recent history. It was first discovered at Research Triangle Institute (RTI) in 1967 when Monroe E. Wall and Mansukh C. Wani isolated the compound from the bark of the Pacific yew tree, *Taxus brevifolia*, and noted its antitumor activity in a broad range of rodent tumors. The structure of paclitaxel was determined in 1970 and since then, Paclitaxel has become an effective tool of doctors in treating patients with lung, ovarian, breast cancer, and advanced forms of Kaposi's sarcoma (Fig 1.7). Paclitaxel falls into the drug category called taxanes and is sold under the tradename Taxol.

History

The history of paclitaxel can actually be traced as far back as 1958 where the Department of Agriculture was commissioned by the National Cancer Institute to collect over 30,000 plant extracts to test for possible anticancer properties. Botanist Arthur S. Barclay, was actually the first scientist to collect bark from Pacific yew trees, in a forest near Mount St. Helens. It was not until much later that Monroe E. Wall discovered that bark extract possessed antitumor qualities, beginning to reveal the tree's hidden treasure. Wall was later join by his colleague Mansukh C. Wani were they set about the task of isolating and purifying plant compounds for anticancer tests. It was not until 1967 the team had isolated the active ingredient, announcing their findings at an American Chemical Society meeting in Miami Beach. In a 1971 issue of the *Journal of the American Chemical Society* Wall and Wani finally published their findings. A young postdoc named Robert

A Holton at Stanford who was studying natural product synthesis, eventually was able to synthesize paclitaxel years later while at Florida State University.

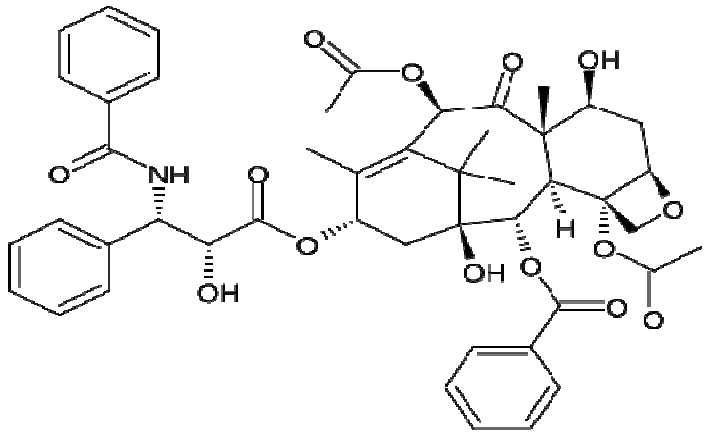


Fig 1.7: Structure of Paclitaxel (Taxol) (55).

Production

An immediate problem that researchers were to face was how to overcome the fact that one patient's treatment may require cutting down 6 full grown trees that are each over 100 years old. As expected this caused a lot of controversy and pressure from environmental groups forced scientists to look for more efficient production processes. Although other paclitaxel-like compounds may be extracted from Pacific and other yew trees, these are not as potent as paclitaxel itself. To address this problem, Hauser Chemical Research of Boulder, Colorado developed a process that allowed the extraction of paclitaxel form the annual "sheds" of the Pacific yew bark.

Initially, a process developed by Hauser Chemical Research of Boulder, Colorado made it practical to extract paclitaxel from the annual "sheds" of Pacific yew bark. This was followed by the process called "Total synthesis", which provided another means of obtaining paclitaxel, using petrochemical-derived starting materials (56). Although this process was very effective, it was not an economically feasible way of manufacturing paclitaxel, in response, semisynthesis was developed. In this process 10-deacetylbaccatin can be extracted in relatively large amounts from various yew-related species and is converted by several steps of organic synthesis into paclitaxel.

Several other processes which were developed to address the same problem include using actinobacteria from which paclitaxel-like compounds can be obtained by fermentation, as well as using cultures of the fungus *Nodulisporium sylviforme* to produce paclitaxel itself.

By far the most environmentally sensitive process is the one that the majority of paclitaxel production today uses. Plant cell fermentation (PCF) technology, involves using calluses of a specific taxus cell line that are then propagated in aqueous medium in large fermentation tanks. The attractive aspect of this process is that the raw materials used for cell growth include sugars, amino acids, vitamins, and trace elements; all of which are renewable nutrients. Direct extraction from the plant cell cultures is then purified by chromatography and isolated by crystallization.

Mechanism of action

The mechanism of action of paclitaxel is as unique as it is effective. The target of paclitaxel is microtubule growth, but what separates it from other drugs that aim to inhibit their function, is that paclitaxel actually hyperstabalizes the microtubules (Fig 1.8). This effectively renders the cytoskeleton of the cell inflexible. Paclitaxel specifically targets the subunits of microtubules, namely β-tubulin. Once bound, by paclitaxel the microtubules are essentially locked in place and unable to disassemble. Since normal cell function requires the assembly and disassembly (dynamic instability) of microtubules in processes such as mitosis, paclitaxel is able to effectively stop cell growth in its tracks.

Cancer cells exhibit many unique features, but one of the most studied is the ability of the cell to divide at a rapid rate. In order to achieve this the cell cytoskeleton must undergo extensive restructuring. Since paclitaxel targets the building blocks required for cellular restructuring, it is a very effective treatment against aggressive forms of cancers. Current

research also supports the hypothesis that paclitaxel not only inhibits cancer cells via microtubule stabilization, but also by binding anti-apoptotic proteins such as Bcl-2 rendering them non functional (57,58,59).

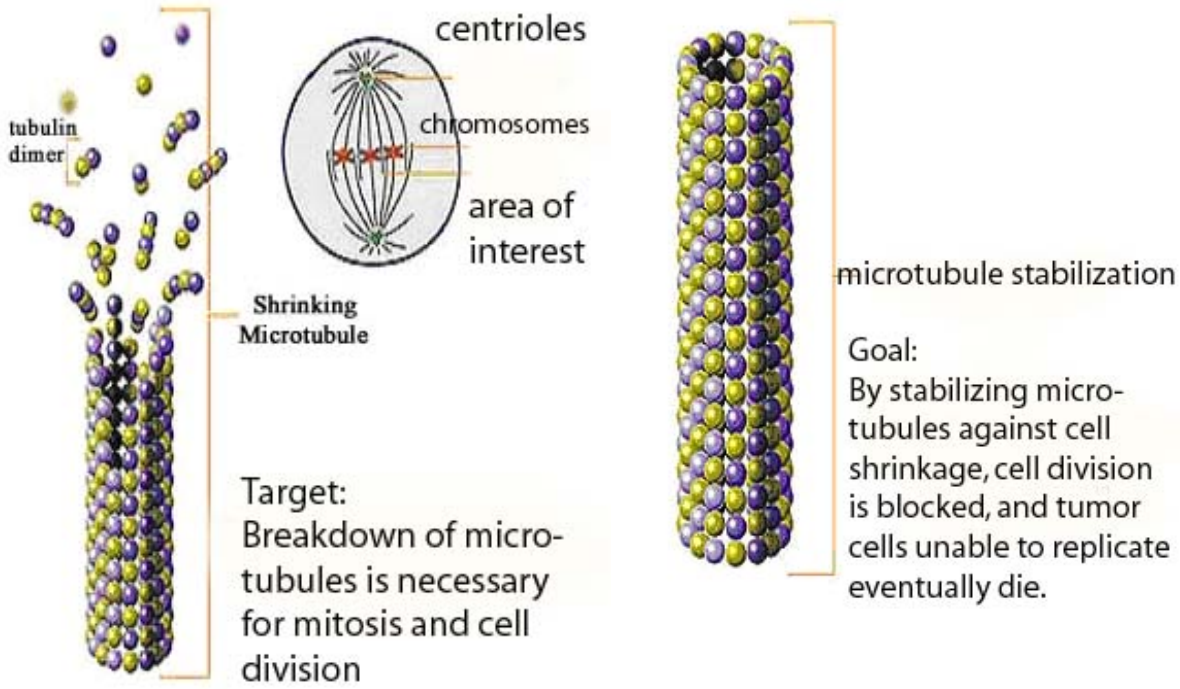


Figure 1.8: The Mechanism of action of Paclitaxel consists of polymerization of tubulin, which results in the formation of abnormally stable and nonfunctional microtubules, thereby inhibiting cellular replication (57)

Bristol-Myers Squibb has held the license to commercialize and market paclitaxel as Taxol since 1993, but in 2005 the Food and Drug Administration (FDA) approved the use of Abraxane (ABI-007) for the treatment of breast cancer. Abraxane is an attractive alternative to Taxol as is it uses paclitaxel bound to albumen instead of the often toxic solvent Chremaphor EL (60,61,62,63)

66cl-4-GFP Murine Mammary Caner Cell Line

The primary cell line presented in this dissertation, 66cl-4-GFP cells were derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone (33,34). 66cl-4 cells were stably transfected with the enhanced green fluorescent protein and selected for a high degree of fluorescence by Dr. Lu-Zhe Sun (UT Health Science Center, San Antonio, TX). The rationale for using this cell line was based on the fact that it has previously been shown to be highly metastatic, with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing microscopic metastases detectable with fluorescent microscopy in the lungs 26 days following s.c. injection of 2×10^5 tumor cells into the inguinal area (24).

Animals

The benefit of using an allograft mouse model is that you do not have the concern of human cells performing differently in a mouse system as they might in a human system. The idea when using this system is in the hope that the results would be equally

applicable to *in vitro* studies. For these studies, Ffemale BALB/cJ mice at 6 weeks of age (20-25 gm body weight) were used. The mice were housed five per cage at the Animal Resource Center at the University of Texas at Austin at 74 ± 2˚F with 30-70% humidity and a 12 h alternating light-dark cycle. Animals were given water and standard lab chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison WI) ad libitum. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Summary

Problems with toxicity in many current chemotherapeutics have accelerated the need for new strategies in the treatment of cancer to achieve effective cancer cell killing without the common side effects that accompany chemotherapy. Vitamin E and several of its derivatives, namely α-TEA are suitable choices as they exhibit low toxicity and have been shown to effect many pro-apoptotic pathways. It is proposed that α-TEA when used in conjunction with a known chemotherapeutic, 9NC and Paclitaxel will be able to decrease the threshold of cell killing, providing greater efficacy and fewer adverse side affects.

This dissertation will focus on the efficacy of α-TEA used alone and in conjunction with 9NC and Paclitaxel its ability to induce DNA synthesis arrest, differentiation and programmed cell death in the 66cl-4-GFP murine mammary cancer cell line *in vitro* and *in vivo*.

Chapter 2 will investigate the effects of an aerosol delivery system using α-TEA alone and in combination with 9-Nitro-Camptothecin (9NC) on 66cl-4-GFP murine mammary cancer cells in the 66cl-4-GFP syngeneic mouse model and investigate the mechanism of apoptosis induced by α-TEA and 9NC.

Chapter 3 aims to elucidate the mechanisms by which α-TEA alone is able to induce apoptosis in 66cl-4-GFP murine mammary cancer cells. Mitochondrial involvement as well as the role of caspases in α-TEA triggered cell death will be discussed.

Chapter 4 will characterize the apoptotic mechanisms of α-TEA in combination with Paclitaxel (Taxol) on 66cl-4-GFP murine mammary cancer cells.

Chapter 5 presents data from an in vivo study using aerosol delivered α-TEA and Paclitaxel alone and in combination when compared individual and untreated control groups.

Chapter 6 Summary and future directions

Chapter 2: α-TEA and 9-Nitro-Camptothecin Alone and in Combination, Induces Caspase Dependent Apoptosis in 66cl-4-GFP Mouse Mammary Tumor Cells *In Vitro* and Reduces Mouse Mammary Tumor Burden and Metastases *In Vivo*.

ABSTRACT

Previous studies have shown that a novel, nonhydrolyzable ether analog of RRR-α-tocopherol, 2, 5, 7, 8-tetramethyl-2R-(4R, 8R-12-trimethyltridecyl)chroman-6-yloxyacetic acid, α-TEA) exhibits anti-tumor activity both *in vitro* and *in vivo* in a mouse mammary tumor model. α -TEA has been shown to be non-toxic and can induce DNA synthesis arrest and apoptosis in cells from BALB/c mouse mammary tumor line 66 clone 4 stably transfected with green fluorescent protein (66cl-4-GFP) in a dose responsive and time dependent manner. Studies presented here show that combinations of α-TEA and 9-Nitro-Camptothecin (9NC) are able to produce increased levels of cell death in 66cl-4-GFP mouse mammary tumor cells *in vitro.* Treatment with α-TEA and 9NC separately and in combination, increased levels of membrane bound FAS, Caspase 8, 9 and p-JNK, suggesting that α-TEA and combination treatments induce cell death in a caspase and mitochondrial dependent manner. To evaluate the role of JNK, protein levels were knocked down with the use of an inhibitor in turn producing a significant decrease in apoptosis suggesting a pivotal role in programmed cell death. To investigate the role of anti-apoptotic players such as c-Flip(L) and Survivin, cells where transfected to overexpress these proteins. Reduced levels of apoptosis were seen in both cases, highlighting the importance of c-Flip(L) and Survivin in tumor cell survival.

In vivo studies using α -TEA and 9NC combination treatments administered by liposomal aerosol, resulted in effective inhibition of tumor growth and lung metastases. Combination treatments reduced tumor volume by 31, 57, 66, 80 and 82 % after 7, 9, 19, 27 and 29 days of liposomal aerosol treatment, respectively. Mice treated with α -TEA or 9NC alone reduced tumor volumes of 51% and 72 % respectively by 29 days. Analysis of fluorescent micrometastatic lung lesions showed that mice treated with aerosol combination results in 95% fewer micrometastatic lung lesions per lobe when compared to mice receiving the aerosol control. Individual treatments with α -TEA or 9NC results in 87% and 86% reduction respectively. α -TEA and 9NC in combination shows 79% fewer lymph node micrometastatic lesions when compared to the control group, (1.62 ± 0.4 vs. 7.9 ± 1.5, $p<.0001$); α-TEA alone shows a 61% reduction when compared to the control (3.04 ± 0.4 vs. 7.9 ± 1.5, $p<.0001$); 9NC alone shows a 22% reduction when compared to the control (6.14 ± 0.75 vs. 7.9 ± 1.5, $p<.71$). The mechanism of α -TEA and 9NC inhibition of tumor growth, *in vivo*, is shown to be via decreasing cell proliferation and inducing apoptosis. The animals treated with α -TEA or 9NC alone, as well as in combination, show an increase in the number of TUNEL positive cells and a reduced number of Ki-67 stained cells. . It should be noted that animals in the 9NC treatment group began to lose weigh towards the end of the study, raising the issue of toxicity. Interestingly, the combination treatment group saw no reduction in body weight suggesting a protective effect given by α-TEA Together this data suggests that individual treatments, as well as combination treatments of α -TEA and 9NC, acting through distinct apoptotic pathways are compelling anti-tumor and anti-metastatic agents.

INTRODUCTION

Breast cancer is the most common type of cancer diagnosed among women in the U.S. In 2007, roughly 42 million mammograms will be performed, of which an estimated 178,480 women will ultimately be diagnosed with invasive breast cancer (1). Breast cancer rates continue to climb with more than 1.2 million breast biopsies performed per year (1). An estimated 40,460 breast cancer deaths in women are expected in 2007, making breast cancer the second leading cause of cancer deaths in women, after lung cancer (1). In recent years, there's been an explosion of life-saving treatment advances against breast cancer, bringing new hope and excitement. Instead of only one or two options, today there's an overwhelming menu of treatment choices that fight the complex mix of cells in each individual cancer. The options; surgery, hormonal (anti-estrogen) radiation therapy and/or chemotherapy. In most cases the latter two options are used following surgery (1).

Until the synthesis of α-TEA, RRR-α-tocopherol succinate (VES, vitamin E succinate) was considered to be the most effective form of vitamin E capable of inhibiting tumor cell growth (63-66). Studies suggested that the tumor inhibitory functions of VES were due to the intact form of VES being maintained, as cleavage of the succinic acid moiety from RRR-α-tocopherol succinate, led to a loss in its ability to exert its anti-cancer abilities (25). As a direct result of these findings there was a need for a novel form containing a non-hydrolyzable ether linkage attaching an acetic acid moiety to RRR-α-

tocopherol. The non-hydrolyzable ether analog of RRR- α -tocopherol, 2, 5, 7, 8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid (abbreviated α -TEA) was developed to meet this need. Previous studies have shown that α -TEA has an intrinsic ability to induce apoptosis in a concentration- and time-dependent manner of murine mammary 66cl-4-GFP tumor cells *in vitro*. It has also been shown to significantly decrease primary tumor burden as well as reduce lung and lymph node micrometastasis in BALB/c mice transplanted with 66cl-4-GFP cells (26). The incorporation of α -TEA into a liposomal aerosol treatment fulfils the desire to make a non-toxic, effective vitamin E-based chemotherapeutic agent that is not only clinically useful but can be delivered in a clinically applicable Fashion (26). Previous studies in our lab have highlighted the potent ability of α -TEA, to induce apoptosis in a wide variety of tumor cells lines namely, human breast (MCF-7, MDA-MB-231, MDA-MB-435), ovarian (A2780, A2780/cp-70), cervical (ME-180), endometrial (RL-952), prostate (LnCaP, PC-3, DU-145), colon (HT-29, DLD-1), lung (A-549) cells while having no adverse effects on normal human mammary epithelial cells (HMEC) or immortalized non-tumorigenic MCF-10A human mammary epithelial cells. (25). There is a need for new strategies in the treatment of cancer that allows for effective cancer cell death without the common side effects that accompany high dose therapies. Vitamin E and several of its derivatives, namely α-TEA, are suitable choices as they exhibit low toxicity and high effectiveness *in vivo* and have been shown to effect many pro-apoptotic pathways (27). It is proposed that α-TEA when used in conjunction with a known

chemotherapeutic, 9NC, will be able to decrease the threshold of cell killing, providing greater capability with fewer adverse side affects.

More than 30 years ago a promising family of anticancer agents were discovered. The camptothecins were first extracted from bark of the Chinese tree, Camptotheca Acuminata, in the early 1960's (39). Camptotheca acuminata is a member of the family Nyssaceae (tupelo family) and is native only to China and Tibet, where it is known as xi shu ("happy tree"). This group of compounds were noted for their ability to induce apoptosis in a wide variety of cancer cell lines, but were still too toxic to be of any practical use. In 1988, in collaboration with scientists from Johns Hopkins and New York Universities, several derivatives of Camptothecin were tested against various types of human cancers transplanted in nude mice. Numerous Camptothecin analogues, including 9NC, demonstrated an unprecedented efficacy against several human tumors including breast, lung, colorectal and ovarian (39,40, 68-69).

The primary target of 9NC is Topoisomerase I, which along with Topoismerase II modulates the topological state of chromatin DNA through the introduction of transient DNA breaks and the removal of excessive super-coils. Whereas Topoisomerase I induces breaks on one strand, Topoisomerase II induces separation on both strands of super-coiled DNA. This process allows for the removal of super-coils, and ensuing enzymatic DNA re-compilation. Since 9NC attaches to the topoisomerase-DNA fragments known as cleavable complexes, it subsequently will inhibit the reuniting process (41-43). Current research supports the idea in which prolonged 9NC induced

stabilization of the Topoisomerase I - DNA complex is connected with conversion of the single strand severance to irreversible double strand severance. This in turn is followed by a pattern of inter-nucleosomal degradation which is indicative of programmed cell death or apoptosis (42).

The success of 9NC has been widely reported in the literature. Preclinical data has shown that it can successfully inhibit human breast cancer xenograf in athymic nude mouse models (70, 71). It has progressed to phase II clinical trials for patients with advanced pancreatic adenocarcinoma, ovarian, tubular or peritoneal cancers, as well as patients with metastatic breast cancer (34,72,73). Several potential hurdles have been reported regarding the use of camptothecin compounds as therapeutic agents, namely bioavailability and toxicity. Issues with bioavailability have been the limiting factor in oral preparation of 9NC, requiring, a more effective delivery vehicle must be considered (74). These problems have been circumvented in part, by continual development of less toxic formulations and more effective delivery methods (71, 75-76). One method of 9NC delivery that is receiving attention is its administration via liposomal aerosol. Current research has shown that this method has been effective while using lower doses with higher bioavailability than those used in other routes of administration against human breast, colon and lung cancer xenographts in various mouse models (71, 78) This delivery system also shows promise when used against experimental pulmonary metastasis in mice (76). The advantages of using liposomal aerosol treatments have been substantiated in toxicological studies in dogs (75) and dose response toxicologic evaluations of cancer patients with advanced lung malignancies (77).

Although several studies have been conducted investigating 9NC's effect on cell growth, differentiation and apoptosis in various cancer cell lines *in vitro* (41-43), there have been only limited studies carried out investigating the effect of 9NC on tumor inhibition in animal models (71), with only one previous study examining the effects of both α-TEA alone and in combination with 9NC in BALB/c mice injected with 66cl-4-GFP mammary cancer cells (79).

The results from this study indicate that combination treatments of α-TEA and 9NC significantly reduce primary mouse mammary tumor growth ($P<0.001$) to a greater degree than either of the individual treatments. Combination treatment also showed a greater ability to inhibit visible lung metastasis, as well as lung and lymph node micrometastasis, while subsequently increasing the number of tumor cells undergoing apoptosis.

MATERIALS AND METHODS

α-TEA and 9-NC *in vivo* and *in vitro* treatments.

α-TEA (FW= 488.8) was prepared for bulk production with high purity (99% pure) and tested for anticancer properties as previously described (26). 9NC (FW= 397.4) was purchased from Chemwerth (Woodbridge, CT). The following antibodies were used: PARP (sc-7150, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GAPDH (made in-house), Fas (sc-716, Santa Cruz), Caspase-9 (sc-7558, Santa Cruz), Caspase- 8 (sc-7890, Santa Cruz), JNK1 (sc-474, Santa Cruz), p-JNK (sc-6254, Santa Cruz). JNK inhibitor

(Calbiochem Corp. San Diego, CA). Caspase 9 inhibitor Z-LEDH-FMK (BioVision Research Products, Palo Alto, CA). Myc-tagged survivin pcDNA3 plasmid, was a kind gift from Dr. J.Q. Chen (Department of Pathology, Molecular Oncology, and Drug Discovery Center & Research Institute, Tampa, Florida), Wild type HA-tagged c-Flip(L) was a provided by Dr. John. Reed (The Burnham Inst. La Jolla, CA). Empty pcDNA vector was purchased from Invitrogen (Carlsbad, CA). Reagents used for morphological analysis of apoptosis were purchased from Boehringer Mannheim (Indianapolis, IN).

66cl-4-GFP Murine Mammary Tumor Cell Line

66cl-4 cells were derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone (33, 78). 66cl-4 cells were stably transfected with the enhanced green fluorescent protein and selected for a high degree of fluorescence by Dr. Lu-Zhe Sun (UT Health Science Center, San Antonio, TX). It has previously been shown that 66cl-4-GFP cells are highly metastatic, with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing microscopic metastases detectable with fluorescent microscopy in the lungs 26 days following s.c. injection of 2 X 10^5 tumor cells into the inguinal area (26). 66cl-4-GFP cells were maintained as monolayer cultures in McCoy's media (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 100 µg/ml streptomycin, 100 IU/ml penicillin, 1 X (vol/vol) non-essential amino acids, 1X (vol/vol) MEM vitamins, 1.5 mM sodium pyruvate, and 50 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO).

Analysis of Apoptosis Via Morphological Evaluation of DAPI-Stained Nuclei.

Apoptosis was determined using previously published procedures (66,87). Cells were plated at 1 x10^5 cells/well in 12-well plates were cultured overnight to permit attachment. The cells were then treated for either 2 or 3 days after which they were collected and floating cells plus scraped-released adherent cells were pelleted by centrifugation for 5 min at 350 X *g*, washed one time with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCL, 10.4 mM Na_2HPO_4, 10.5 mM KH_2PO_4; pH 7.2), and stained with 2 µg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim, Indianapolis, IN) in 100% methanol for 15 min at 37˚C. Cells were viewed at 400X magnification with a Zeiss ICM 405 fluorescent microscope using a 487701 filter. Cells in which the nucleus contained condensed chromatin, or cells exhibiting fragmented nuclei, blebbing, were scored as apoptotic. Data are reported as percentage of apoptotic cells per cell population (i.e. number apoptotic cells/total number of cells counted). Three different microscopic fields were examined and 200 cells counted at each location for a minimum of 600 cells counted per slide. Apoptotic data are presented as mean ± S.D. for three independently conducted experiments.

Western Immunoblot Analysis

66cl-4-GFP cells were plated at a density of 1.5 x 10^5 cells/ml in T-25 flasks (10 ml/flask) and allowed to adhere overnight. Next, the media were removed and replaced with experimental media containing treatments of α-TEA (10µg/ml), 9NC (125ng/ml), combination and VEH. Cells were treated for either 6, 15, 24 or 48 hours and then

collected. by scraping to combine floating and adherent cells, and then pelleted by centrifugation at 350 g.

To determine presence and levels of protein within the cell, whole cell lysates were prepared as previously described (66,87). Cells were then washed in PBS to remove any remaining media and lysed with lysis buffer (1x PBS, 1% NP40, 0.5% sodium deoxyxholate, and 0.1% SDS) added to 1µg/ml aprotinin and leupeptin, 1mM DTT and finally 2 mM sodium orthovadate. The lysis mixture is incubated on ice for 30 minutes. Lysates are then centrifuged at 15,000 x g for 10 minutes at 4 0 C and then protein concentrations determined following the Bio Rad Dye Binding protein assay. 50µg of protein was used per lane and either run on a 10%, 12% or 15% SDS-page gel under reducing conditions (26,79). Protein were then probed using their respective antibodies.

Caspase Inhibitors

66cl-4-GFP cells were plated at a concentration of $1.5x10^5$ cells/well in 12 well plates and then treated with one of the following caspase inhibitors: Caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-VAD-FMK) were all purchased from BioVision Research Products (Palo Alto, CA), at 2µM for 2 hours. They were then treated with 10µg/ml α-TEA, 125ng/ml or combination for three days. To determine the percentage of cells undergoing apoptosis, DAPI analysis was used.

JNK Inhibitor

66cl-4-GFP cells were plated at $1.5x10^5$ cells/well in 12 well plates and then treated with

40 nM of JNK inhibitor II (Calbiochem-Novabiochem Corp. San Diego, CA), or DMSO vehicle for 2 hours prior to treatment with α-TEA (10μg/ml), 9NC (125ng/ml) for 48 hours. To determine the percentage of cells undergoing apoptois, DAPI analysis was used.

Transient Transfection

66cl-4-GFP cells were plated at a concentration of $1.5x10^5$ cells/ml in 12 well plates and then allowed to adhere overnight. Prior to transfection with Myc-tagged survivin, HA-tagged c-Flip(L) or vector controls, cells were washed with serum free media. Briefly, 0.7μg of DNA was added to 50μl serum free media containing 4μl of Plus (Invitrogen, Carlsbad, CA) reagent which was then incubated for 15 mins (mix 1). 2μl of LipofectAMINE reagent was added to 50μl serum free media and then combined with mix 1and incubated for a futher 15 mins. Cell were then allowed to incubate for 8 hours after which the media was replaced with normal culture media overnight. Cells were then treated with individual or a combination of α-TEA (10μg/ml) and 9NC (125ng/ml) for 48 hours. To determine the percentage of cells undergoing apoptois, DAPI analysis was used. To investigate cellular protein levels cells were then treated with individual or a combination of α-TEA (10μg/ml) and 9NC (125ng/ml) for 6,15,24 and 48 and then probed using western immunoblot.

Statistical Analysis of Data

Statistical analysis of the data was performed using Prism software version 4.0

(Graphpad Prism, San Diego, CA) were a level of $P<0.05$ was considered to be statistically significant. Apoptosis data was analyzed using a one way ANOVA followed by a Tukeys's post-hoc test.

Animals

Female BALB/cJ mice at 6 weeks of age (20-25 gm body weight) were purchased from Jackson Labs (Bar Harbor, ME). The mice were housed five per cage at the Animal Resource Center at the University of Texas at Austin at 74 ± 2˚F with 30-70% humidity and a 12 h alternating light-dark cycle. Animals were given water and standard lab chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison WI) ad libitum. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Tumor Cell Inoculation

66cl-4-GFP cells were harvested by trypsinization, collected by centrifugation, and re-suspended at a density of 2×10^5 cells/100 µl in McCoy's media, that contained no supplements. Mice were injected with 2×10^5 cells/ml/100µl in the inguinal area at a point equal distance between the 4^{th} and 5^{th} nipples on the right side using a 23 gauge needle. 40 mice (10 mice/group) were assigned to aerosol α-TEA, aerosol 9-NC, aerosol α-TEA + aerosol 9NC, or aerosol control group so that the mean tumor volume for the four groups was closely matched. Treatments began ten days following tumor cell inoculation. Tumors were measured using calipers every other day, and volumes were

calculated using the formula: volume (mm^3) = [width $(mm)^2$ X length (mm)] /2 (83). Body weights were determined on a weekly basis to evaluate possibility of drug toxicity.

Preparation of α-TEA and 9-NC Liposomes for Delivery by Aerosol

An α -TEA/liposome ratio of 1:3 (w/w) was prepared as described previously (26). Briefly, the lipid [1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC); Avanti Polar-Lipids, Inc., Alabaster, AL] at a concentration of 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX), then sonicated to obtain a clear solution. α -TEA at 40 mg/ml was dissolved in tertiary-butanol and vortexed until all solids were dissolved. The two solutions were combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α -TEA/liposome. 9-NC (100 mg/ml) was dissolved in DMSO and warmed to 37˚C. DLPC (100 mg/ml) was dissolved in tertiary-butanol. Drug and liposome solutions were mixed together at a 1:50 (w/w) ratio. Both treatments were mixed by vortexing, frozen at –80º C for 1-2 h, and lyophilized overnight to a dry powder prior to storing at –20˚C. Each treatment vial contained either 75mg of α –TEA or 2mg of 9-NC.

Aerosol Delivery

Aerosol was administered to mice as previously described (26, 78, Fig. 2.1). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 L/min airflow was used with an AeroTech II nebulizer (CIS-US, Inc. Bedford, MA) to generate aerosol. The particle size of α -TEA liposome aerosol

discharged from the AeroTech II nebulizer was determined by the Anderson Cascade Impactor to be 2.01 µm mass median aerodynamic diameter (NMAD), with a geometric standard deviation of 2.04. About 30% of such particles when inhaled will deposit in the respiratory tract of the mouse and the remaining 70% will be exhaled (78). In this study the level of α-TEA and 9NC were doubled compared to previous studies using this cell line (79). Prior to nebulization, two vials of α -TEA/lipid powder (75 mg/vial) and one vial of 9-NC/lipid powder (2mg/vial) were brought to room temperature, then reconstituted by adding 3.75 ml and 10 ml distilled water, respectively, to achieve the final desired concentration of 20 mg/ml α -TEA and 200 µg/ml 9-NC. The mixtures were allowed to swell at room temperature for 30 min with periodic inversion and vortexing, and then added to the nebulizer. Mice were placed in plastic cages (7 x 11 x 5 in.) with a sealed top in a safety hood. Aerosol entered the cage via a 1inch accordion tube at one end and discharged at the opposite end, using a one-way pressure release valve. The total amount of α –TEA used was therefore 150mg/cage/day and 9NC was 2mg/cage/day. Animals were exposed to aerosol until all α -TEA / liposome or 9-NC / liposome was aerosolized (approximately 25 min). For α -TEA + 9-NC combination treatments, α -TEA was administered by aerosol 8 hours prior to delivery of 9-NC by aerosol each day. Approximately 72µg of α-TEA and 0.8µg 9NC was deposited in the lungs of each mouse per day.

Lung and Lymph Node Metastasis.

Visible macroscopic metastases were counted in all fivelung lobes at time of sacrifice. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; 200 X magnification) as previously described (26). For micrometastatic analyses, the left lung lobe was flattened and the top and bottom surfaces were scored for fluorescent microscopic metastases. Fluorescent microscopic metastases were scored by size into three size grouping: < 20 µm, 20-50 µm, and >50 µm. Based on a typical 66cl-4-GFP tumor cell size of 10-20 µm in diameter, the < 20 µm grouping is thought to represent solitary cells; the 20-50 µm grouping two to five cells; and the > 50 µm grouping microscopic metastases of greater than two to five cells

Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling Assay for Detection of Apoptosis *in Vivo*.

Deparaffinized sections (5 µm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag In Situ Apoptosis Detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Nuclei that stained brown were scored as positive for apoptosis and those that stained blue were scored as negative. At least sixteen 400X microscopic fields were scored per tumor. Data are presented as the mean ± S. E. number of apoptotic cells counted in ten separate tumors from each group.

Ki-67 immunohistochemical staining for detection of proliferation *in Vivo*.

Deparaffinized sections (5 µm) of tumor tissue were used to assess proliferation, using antibody to the Ki-67 antigen, which is a nuclear antigen expressed in proliferating cells and which serves as an indicator of the number of cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 minutes followed by washing with PBS. Rabbit serum (10 %) in PBS was applied to 5 micron tumor tissue sections in order to block non-specific antibody binding, prior to incubating with primary antibody (rat-anti-mouse Ki-67 antibody; 1:200 dilution, DAKO Corp., Carpinteria, CA) overnight at 4°C. After primary antibody incubation, washed slides were incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 minutes at room temperature. Sections were then incubated with avidin-biotin complex (ABC-HRP, Vector Laboratories) for 30 minutes at room temperature. Immunoreactivity was visualized via incubation with di-aminobenzidine dihydrochloride (DAB). Slides were lightly counterstained with hematoxylin. Ki-67 positive stained (brown) cells were counted in five separate fields per sample.

CD31 staining for determination of blood vessel formation *in vivo*.

Immunohistochemistry was used to assess the presence of the endothelial antigen CD31 as an indicator of small capillaries in primary tumor tissue. Deparaffinized tissue sections were pre-treated with 0.06% Protease Type XXIV (Sigma) for 10 minutes at room temperature prior to incubation with CD31 (PECAM) antibody (PharMingen, San

Diego, CA) at a 1:400 dilution overnight at 4 °C. Detection utilized the Tyramide Signal Amplification Biotin System - Peroxidase (PerkinElmer Life Sciences, Boston, MA) with di-aminobenzidine dihydrochloride development. For contrast, the sections were lightly counterstained with hematoxylin. Entire tumor sections were scored for CD31 stained vessels (brown), and adjusted for tumor size, by dividing number of CD31 vessels by length X width of tumor tissue mounted on slide.

Statistical Analyses

Animal numbers for experiments were determined by power calculations derived from data generated by preliminary pilot studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance using SPSS (SPSS, Inc, Chicago, IL). Difference in number of fluorescent microscopic metastases per group was determined using the Mann-Whitney rank test using Prism software version 4.0 (Graphpad, San Diego, CA). A level of $P < 0.05$ was regarded as statistically significant.

Results

Individual and Combination Treatments of α-TEA and 9NC induce apoptosis in a caspase and JNK dependent Fashion in 66cl-4-GFP Murine Mammary Cancer Cells *In Vitro.*

Cells were treated with α-TEA (5 or 10 μg/ml) or 9NC (62 or 125ng/ml) for three days, showed increased levels of apoptosis (2.8, 10, 3.7 and 6.2 fold respectively) via DAPI

staining when compared to ethanol or DMSO controls. Combination treatments of α-TEA (5μg/ml) with 9NC (62 or 125ng/ml) showed 6 and 7.5 fold increase in apoptosis when compared to EtOH/DMSO control (Fig 2.2A). α-TEA (10μg/ml) with 9NC (62 or 125ng/ml) showed 6.7 and 10.5 fold increase when compared to EtOH/DMSO control (Fig 2.2B). Combination treatments with α-TEA (5μg/ml) and 9NC (62,125ng/ml) displayed 4.9 and 6.1 fold higher levels of apoptosis when compared to α-TEA alone (Fig 2.2A). α-TEA (10μg/ml) and 9NC (62ng/ml) did not show any significant increase in apoptosis, whereas α-TEA (10μg/ml) and 9NC (125ng/ml) exhibited a 1.5 fold increase when compared to α-TEA alone (Fig 2.2B).

Caspases, Fas and JNK Play Key Roles in Apoptosis in 66cl-4-GFP cells Following Treatments with α-TEA and 9NC

To gain a better understanding of how α-TEA and 9NC Cotreatments are able to induce apoptosis in 66cl-4-GFP cells, FAS and caspase involvement was investigated. Cotreatments using α-TEA (10μg/ml) with 9NC (125ng/ml) did not increase overall cellular levels of FAS but was able to induce an increase in cell membrane Fas starting at 15 hour and reaching peak levels at 48 hours. α-TEA and 9NC together were able to induce caspase 8 and 9 cleavage starting at 15 hours. PARP cleavage was seen at 15 hours and continued through 48 hours following cotreatment (Fig 2.3A).

In order to further determine the involvement of caspases 8 and 9 in cotreatment induced apoptosis, cells were pre-treated with caspase specific inhibitors and then levels of apoptosis were measured. Addition of inhibitors to caspase 8 and 9 significantly reduced

apoptosis in treated cells with α-TEA (10μg/ml) and 9NC (125ng/ml) cotreatments. (Fig. 2.3B). This data suggest that α-TEA and 9NC cotreatments induced apoptosis is caspase dependent, specifically working through caspases 8 and 9.

To elucidate the role of JNK we first investigated the effect of a JNK inhibitor on the ability of α-TEA and 9NC cotreatments to induce apoptosis. The use of the JNK inhibitor was able to significantly reduce the number of apoptotic cells as determined via DAPI staining. This data suggests that JNK does play a significant role in apoptosis induced by cotreatments in 66cl-4-GFP cells (Fig 2.4A). Knowing that cotreatments were able to induce apoptosis using JNK, we next investigated the effect of α-TEA and 9NC on activated JNK (p-JNK). Starting at 15 hours separate and cotreatments were able to up-regulate p-JNK and continued to do so through 48 hours. Total JNK levels were not modulated (Fig 2.4B).

c-Flip(L) and Survivin play key anti-apoptotic roles in cells treated with α-TEA and 9NC Cotreatments

Anti-apoptotic proteins survivin and, c-Flip(L) are potent regulators in ensuring tumor cell survival. To investigate their importance in α-TEA and 9NC induced apoptosis, 66cl-4-GFP cells overexpressing pcDNA survivin and pcDNA c-Flip(L) were treated with α-TEA (10μg/ml), 9NC (125ng/ml) and combination treatments for 72 hours and evaluated for apoptosis via DAPI staining. Increased levels of Survivin and c-Flip(L) were confirmed via western immunoblot (Fig 2.5A). Apoptotic levels were significantly reduced in cells expressing either pcDNA c-Flip(L) (63%) or pcDNA survivin (61%) ($P < 0.001$, $P < 0.001$), when compared to cotreated treated control vector (Fig 5B).

Individual and cotreatments of α-TEA and 9NC were able to decrease anti-apoptotic proteins survivin and c-Flip(L) over 6,15,24 and 48 hours (Fig 2.5C). Cotreatments showed a greater decrease in both of these proteins when compared to individual treatments at selected time points. There was a 10.3 fold decrease in survivin at 48 hours with the cotreatment, whereas α-TEA and 9NC exerted a more beneficial effect on c-Flip(L) starting at 6 hours (5.6 fold decrease).

α-TEA and 9NC alone and in combination when delivered via liposomal aerosol have an inhibitory affect on tumor burden, in 66cl-4-GFP cells allografted into BALB/c mice.

Mean tumor volumes in the α-TEA, 9-NC and combination treatments were significantly lower when compared to the control group ($P<0.001$; means ±SE 489±153 mm^3; 350±81; 229±41 mm^3 and 727±278 mm^3 respectively) during 29 days of treatment (Fig. 2.6A). The combination treatment group did not show a mean tumor volume that was significantly lower than individual α-TEA or 9-NC ($P<0.2$; mean ±SE 489±153 $mm^3$and $P<0.5$ 350±81 mm^3) groups, while α-TEA and 9-NC treatment groups showed tumor volumes that were not significantly different from each other ($P<0.5$; mean ±SE 489±153 $mm^3$and 350±81 mm^3).

Animal Weights Over Duration of Study

Animal weight for the α-TEA, cotreatment and control groups appear to show no significant overall difference using the Mann-Whitney rank test (Fig 2.6B). It should be

noted that the 9NC treatment group showed a downward trend in the average weight for the group at weeks 3 and 4. Body weight in 9NC only treated mice at completion of the study were significantly decreased when compared to control or other treatment groups.

α-TEA + 9NC Combination Treatment Reduced Visible Lung Metastasis.

When the animals were euthanized at the completion of the study, all five lung lobes from each mouse were examined. Visible macroscopic metastasis were observed in five out of ten mice in the control group with a total of twenty two visible metastasis being detected. Four out of ten mice in the α-TEA and 9NC combination group showed visible metastasis. The total number of macroscopic lung tumor foci (10) were significantly reduced in comparison to the control (22) (Table 2.1).

α-TEA and 9NC alone and in combination have an inhibitory effect on lung and lymph node micrometastatic lesions in BALB/c mice when delivered via liposomal aerosol.

Green fluorescing microscopic lung micrometastatic lesions were counted in control and three treatment groups. Analyses of the data showed a significant decrease in total number of microscopic lung metastases in all three treatment groups in comparison to the control group (Fig 2.7A). In the α-TEA, 9NC and combination treatment groups the mean numbers of microscopic lung metastases were, ($P<0.0001$; 7.7 ± 2.1; 8.4 ± 2.3; and 2.7 ± 1.2 respectively) significantly lower when compared to the aerosol control group

(63.4 ± 11.5). Combination treatments showed significantly lower total levels of microscopic lung metastases when compared to individual treatments.

Green fluorescent microscopic metastases in the axillary and brachial lymph nodes from each treatment group were counted and the data is represented as the number of metastatic lesions per lymph node per group. Both the α-TEA and co-treatment groups exhibited a significant reduction in metastases (P<0.0001; 3.3 ± 0.35; and 1.62 ± 0.43) when compared to the control group (7.86 ± 1.55; Fig. 2.7B). Cotreatments were able

Identification of TUNEL Positive Stained Nuclei Indicating Induction of Apoptosis

TUNEL staining of 5 µm tumor sections were used to evaluate the induction of apoptosis by the treatment groups *in vivo*. The mean ± SE number of TUNEL positive cells/field for α-TEA, 9NC and α-TEA+9NC treatments were 3.7 ± 0.27, 2.6 ± 0.16 and 4.6 ± 0.24, compared to the tumors from the aerosol control group which were 0.8 ± 0.15 TUNEL positive cells per field (P<0.0001). The combination group showed significantly more apoptotic cells per tumor than any of the other groups (combination treatment vs. α-TEA, P< 0.02; combination treatment vs. 9NC, P<0.0001, Fig. 2.8A).

α-TEA and 9NC alone and in combination have an inhibitory effect on cell proliferation *In Vivo*.

The effects on cell growth were determined by examination of tumor sections taken from each of the three treatment groups plus control which were then subject to

immunohistochemical staining for the nuclear antigen Ki-67, a biomarker for proliferation. The mean ± SE number of positive cells per field for tumors taken from each group were: α-TEA ($P<0.001$; 150.8 ± 20.8); 9NC ($P<0.03$; 194.8 ± 20.5) and α-TEA+9NC ($P<0.0001$; 114.5 ± 13.5); compared to tumors from the aerosol control group had a mean ± SE of 268.1 ± 25.1. The Ki-67 staining of tumor sections from mice treated with α-TEA+9NC did not show a significant difference from α-TEA or 9NC treatment groups (Fig. 2.8B).

α-TEA and 9NC alone and in combination showed no significant inhibitory effect on tumor angiogenesis.

Effects of α-TEA, 9NC and α-TEA+9NC treatment on blood vessel density was determined via staining for the CD31 (PECAM-1) endothelial cell marker. Although mean number of CD31 positive stained cells were reduced in 9NC and combination treatment groups, treatment groups displayed no significant difference in number of blood vessels per tumor section when compared to the control groups (Fig. 2.8C).

Discussion

In these studies we demonstrate the effectiveness of a novel vitamin E derivative known as α-TEA used in combination with a known chemotherapeutic agent 9NC in inhibiting primary tumor burden in a syngeneic mouse model. The data show that α-TEA used as a single treatment and when used in combination with 9NC produces a significant decrease in primary tumor burden as well as lung and lymph node micrometastasis when

compared to the control group. Furthermore single and combination treatments using α-TEA and 9NC, show a marked decrease in the number of proliferating cells in the tumor tissue and an increase in the number of TUNEL positive apoptotic cells. The presence of a 3gram differential between the heaviest and lightest weighing groups may suggest a toxic effect of too high concentration of 9NC. The fact that the combination group showed no obvious signs of toxicity as determined by the animals' weights, may imply that α-TEA may be providing a protective effect combating the toxicity of 9NC. The data reported here shows that α-TEA was ineffective in reducing visible lung metastasis when compared to the control group. This result is inconsistent with all other studies performed to date, which effectively show that α-TEA alone significantly reduces visible lung metastasis (26,33,79, 82).

The advantages of using a compound such as α-TEA that has no known adverse side effects with 9NC in a combined therapy is evident. These finding are consistent with previous studies that show α-TEA to be an effective chemotherapeutic agent when combined with other drugs (cisplatin against ovarian cancer and celecoxib against breast cancer) (82, 83), and support the need for continued studies with α-TEA. The data implies that the concentration of 9NC used in this study may have caused toxicity in the animals, as suggested by the reduced animal weights for 9NC treated animals. In contrast, when the same concentration of 9NC was used in conjunction with α-TEA, there appeared to be no obvious toxic effect as exhibited by no weight loss in these animals. This observation suggests a unique characteristic of α-TEA in that it can act in a

protective capacity when used alongside potentially toxic chemotherapeutics to reduce the toxicity of these agents.

The *In vitro* the mechanism by which α-TEA and 9NC combination treatments are able to induce apoptosis is appears to include FAS, is caspase dependent and signals through JNK. It is hypothesized that the method of apoptotic induction involves both the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is characterized by activation of membrane bound death receptor found on the cell surface. This type of pathway requires key players including FADD and the activation of caspase-8. The intrinsic apoptotic pathway is defined by JNK mediated cytochrome *c* release from the mitochondria into the cytosol, where it then can bind Apaf-1, and procaspase-9, causing activation of caspase-9 and eventually apoptosis. Studies carried out in our lab show that inhibition of the Fas receptor in the 66cl-4-GFP cell line, causes a significant decrease in cells undergoing apoptosis when treated with α-TEA or 9NC. We have seen both activation of effector caspases and key intrinsic players such as Bax and cytochrome c (data not published). This hypothesis gains support from studies that suggests along with caspase-8 and 9 activation, Bax, Bid and cytochrome c play an important role in 9NC induction of apoptosis (84, 85). Kharbanda *et al* give further weight to this argument by reporting that one way 9NC is able to induce apoptosis is via activation of c-jun. In their studies, it was shown that an increase in c-jun expression occurs in conjunction with internucleosomal DNA fragmentation, a characteristic which is indicative of programmed cell death (86).

In summary the data reported here reiterates earlier studies that show that α-TEA when used in combination with 9NC is an effective anticancer agent. Not only can it induce significant levels of apoptosis *in vitro* but also that it can significantly reduce primary tumor burden, decrease lung and lymph node micrometastases, and increase the number of apoptotic positive cells *in vivo*. The data suggests that administration of α-TEA, alone and in combination with 9NC, via a clinically relevant system holds great promise as an effective way to treat aggressive mouse mammary tumor model. It is hoped that these encouraging results will transfer equally well to the treatment of human breast cancers.

Acknowledgements

We would like to thank the Histology Core Facility for preparation of H & E and immunohistochemically stained tissues and the Director of the Biostatistics and Data Processing Core, Dr. Dennis A. Johnston and Dr Howard Thames, for their help with statistical analyses (National Institute of Environmental Health Sciences Center Grant ES 07784; University of Texas M. D. Anderson). Dr. Vernon Knight's and Dr. Brian Gilbert's laboratory at Baylor College of Medicine, Houston, TX for the method of aerosol preparation and output analysis and Dr. Lu-Zhe Sun's laboratory at UT Health Science Center, San Antonio, TX for the use of their microscope.

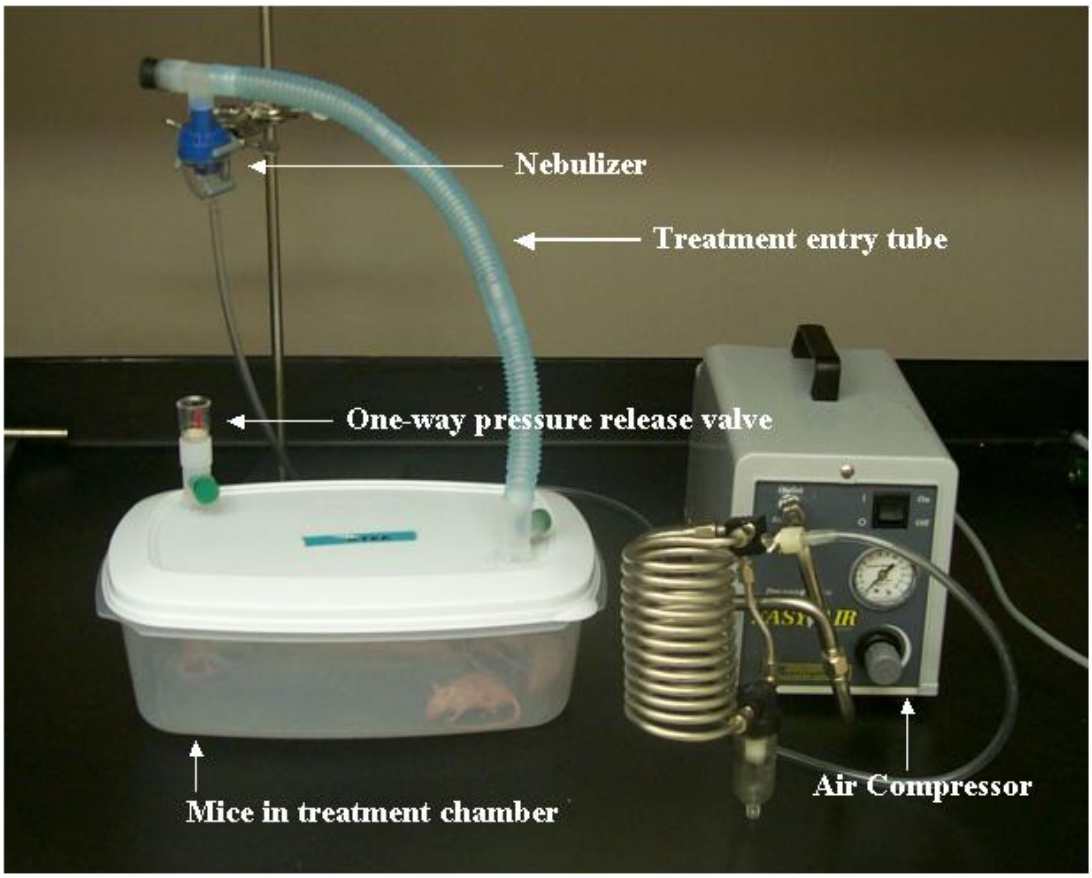
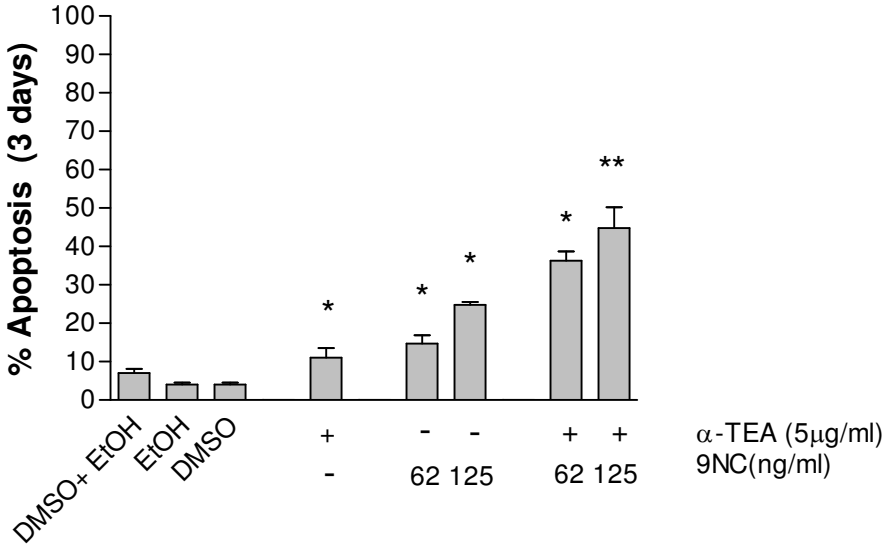


Figure 2.1: Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 L/min airflow was used with an AeroTech II nebulizer (CIS-US, Inc. Bedford, MA) to generate aerosol. The aerosol was channeled to the treatment cage via accordion tubing. Aerosol was allowed to discharge from an opening in the opposite end of the cage via a one way valve.

A



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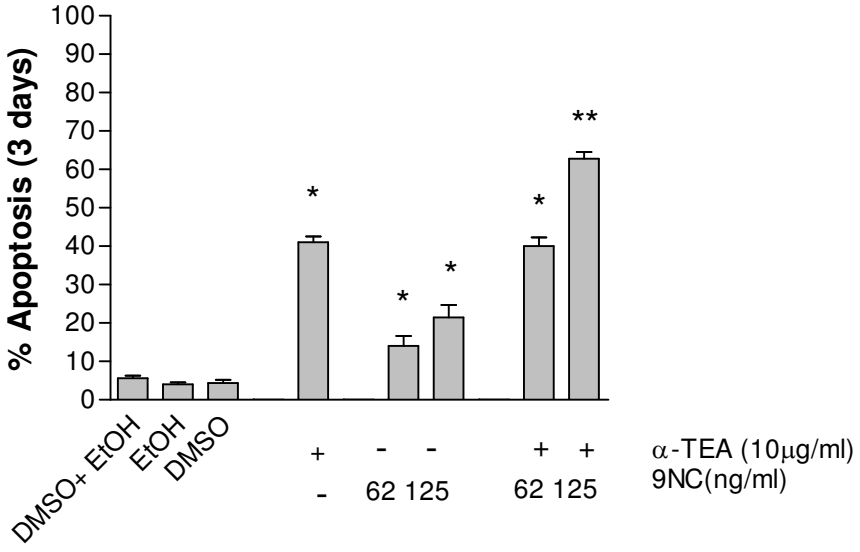


Figure 2.2: α-TEA and 9NC alone and in combination induced apoptosis in 66cl-4-GFP cells. (A and B) Cells were treated with EtOH, DMSO or both and served as untreated controls, or treated with α-TEA (5, 10μg/ml) or 9NC (62,125ng/ml) or combinations and incubated for 3 days. After collection cells were analyzed for apoptotic levels using DAPI staining. Data presented are mean ± S.D.of three independent experiments.
* significantly different from control group; P<0.05
** significantly different from individual treatment groups; P<0.05

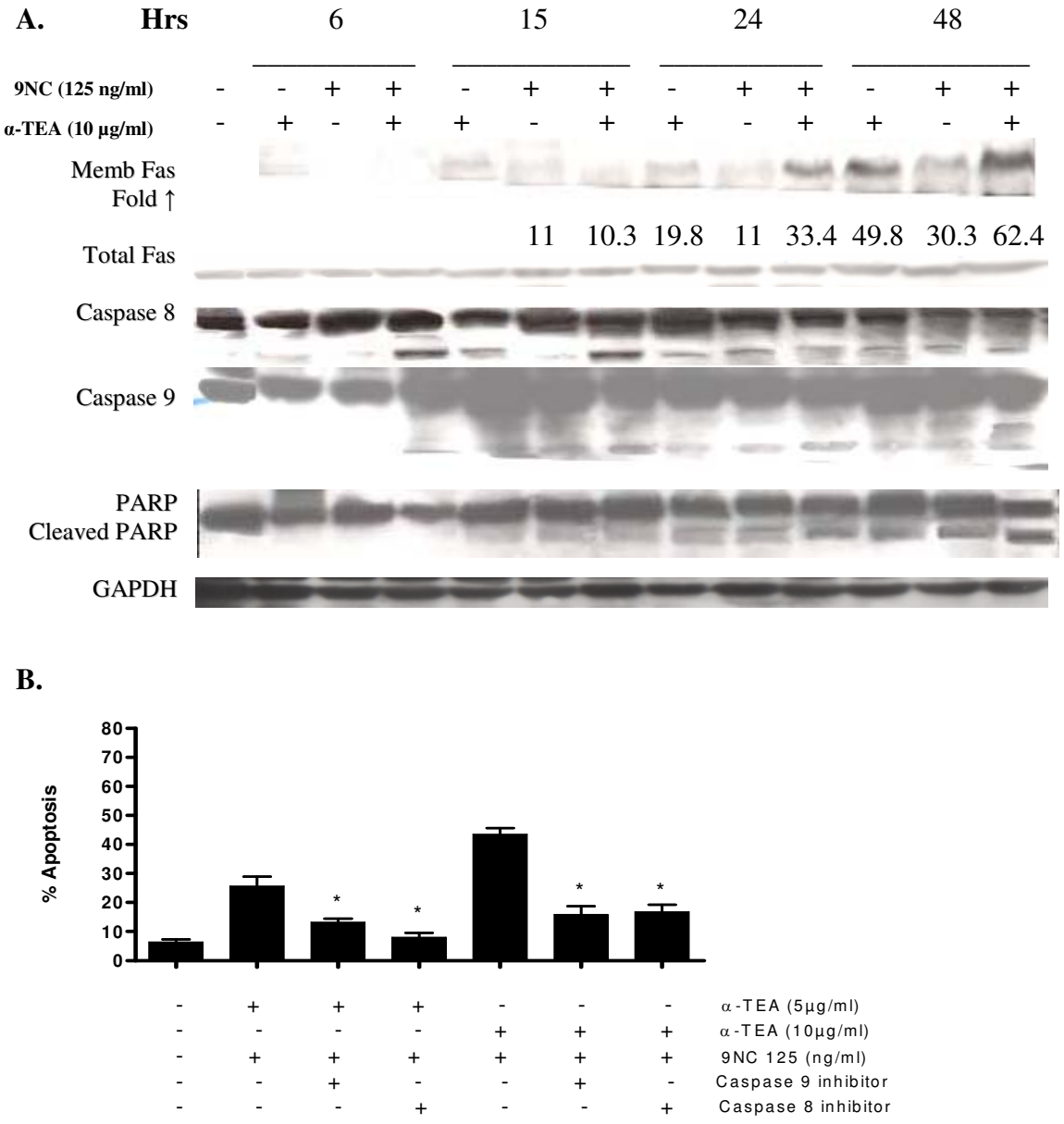
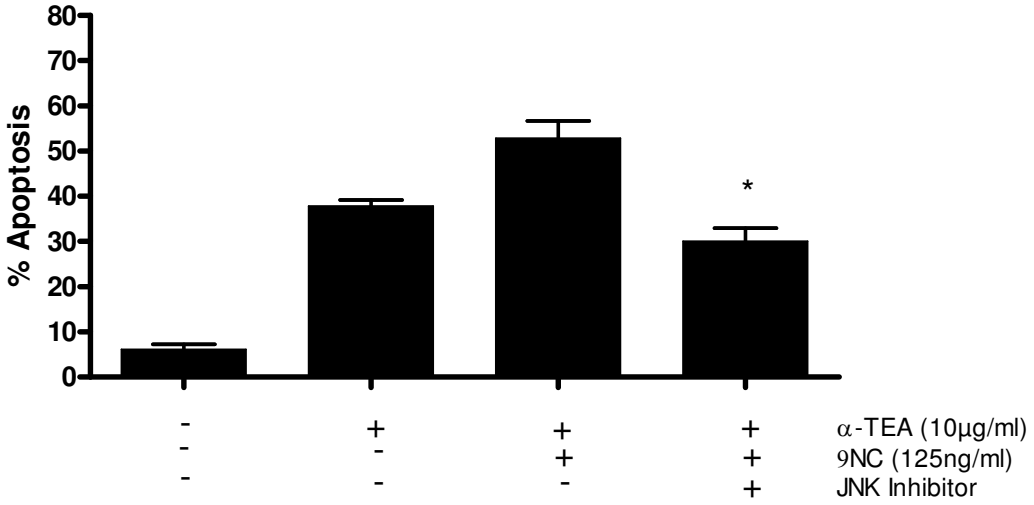


Figure 2.3: (A) α -TEA and 9NC in combination induced up regulation of membrane bound FAS, cleavage of caspase 8,9 and PARP. Cells were treated with VEH, α-TEA (10µg/ml), 9NC (125ng/ml) or combinations and incubated for 6, 15,24 and 48 hours. Protein levels were determined via western immunoblot analysis using the respective antibodies. (B) Cells treated with an inhibitor to caspase 9 (Z-LEDH-FMK) and caspase 8 (Z-IETD-FMK) showed reduced apoptotic levels when treated with α -TEA and 9NC. 66cl-4 GFP cells were pretreated with the caspase inhibitors (2 µM) for 2 hours prior to treatment with α -TEA (10µg/ml), 9NC (125ng/ml) or combinations for 3 days. Apoptotic cells were determined via DAPI staining.
* significantly different from control group; P<0.05

A.



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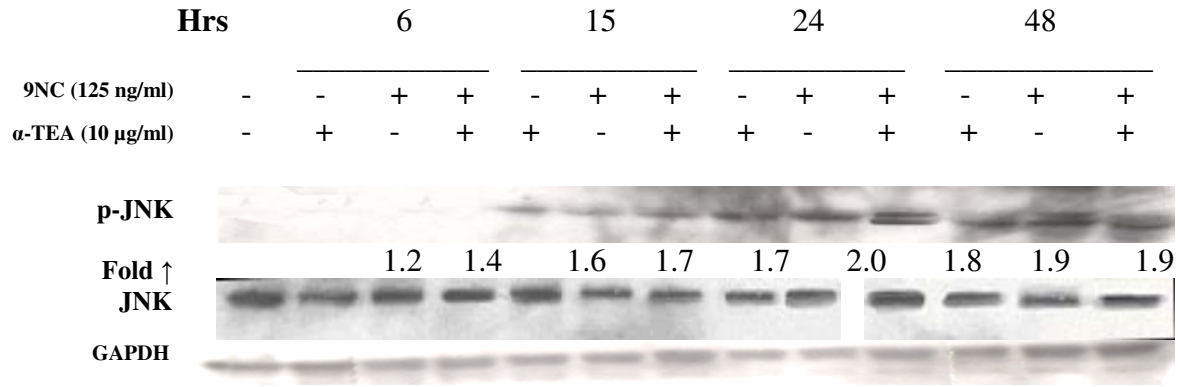


Figure 2.4: Inhibition of JNK reduced apoptosis in cells treated with α –TEA and 9NC in combination. (A) Cells were pretreated with 40 nM JNK inhibitor II or DMSO control for 2 hours followed by treatment with 20µg/ml α –TEA, 125ng/ml 9NC or combination treatment for 2 days. Apoptotic cells were determined via DAPI staining. (B) Cells were treated with VEH, α-TEA (10µg/ml), 9NC (125ng/ml) or combinations and incubated for 6, 15,24 and 48 hours. Protein levels were determined via western immunoblot analysis using the respective antibodies. α -TEA and 9NC alone and in combination up-regulate p-JNK. Data are representative of three independent experiments.
* significantly different from cotreatment group; $P<0.05$

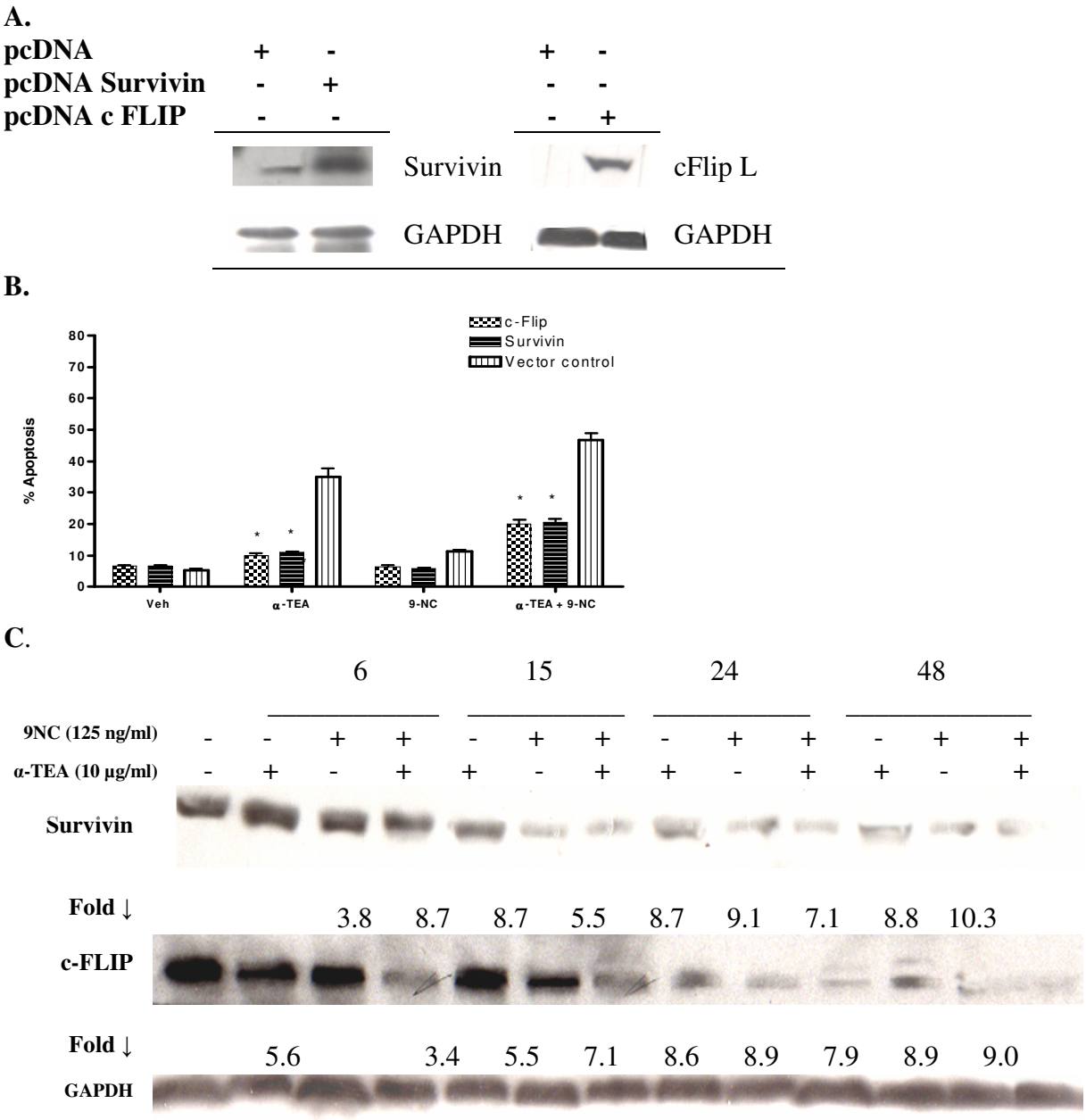
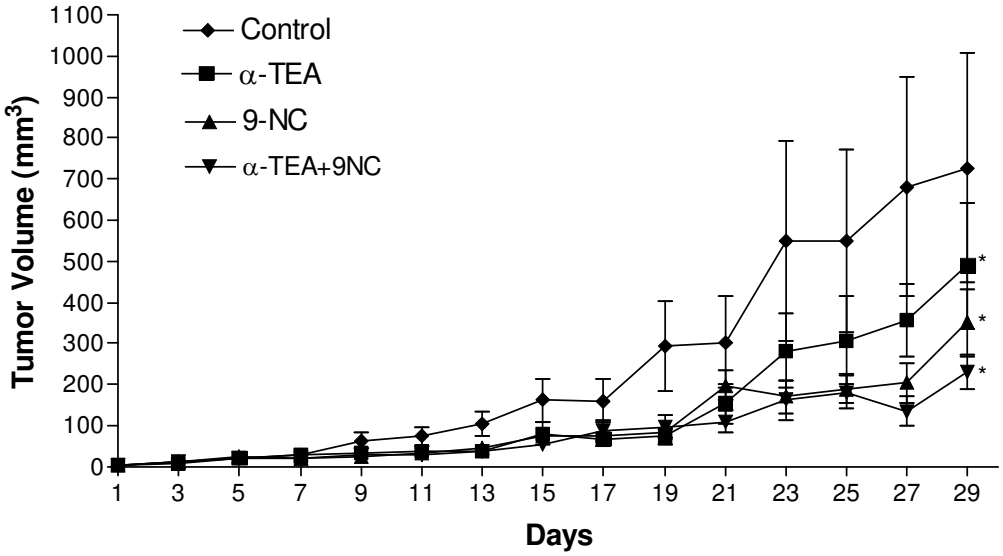


Figure 2.5: Flip and survivin play a key role in preventing apoptosis induced by α -TEA and 9NC cotreatment (A) Western immunoblot shows a decrease in Survivin and c-Flip(L) after individual and cotreatments with α-TEA and 9NC (10µg/ml and 125ng/ml respectively) for 6,15,24 and 48 hours. (B) Cells were transfected with c-Flip(L), survivin or control over-expression vectors and then treated with α-TEA (10µg/ml) or 9NC (125ng/ml) alone and in combination for 3 days. Percentage of apoptotic cells was determined via DAPI staining. (C) Western immunoblot showing overexpression of Survivin and c-Flip(L). Data are representative of three independent experiments.
A.



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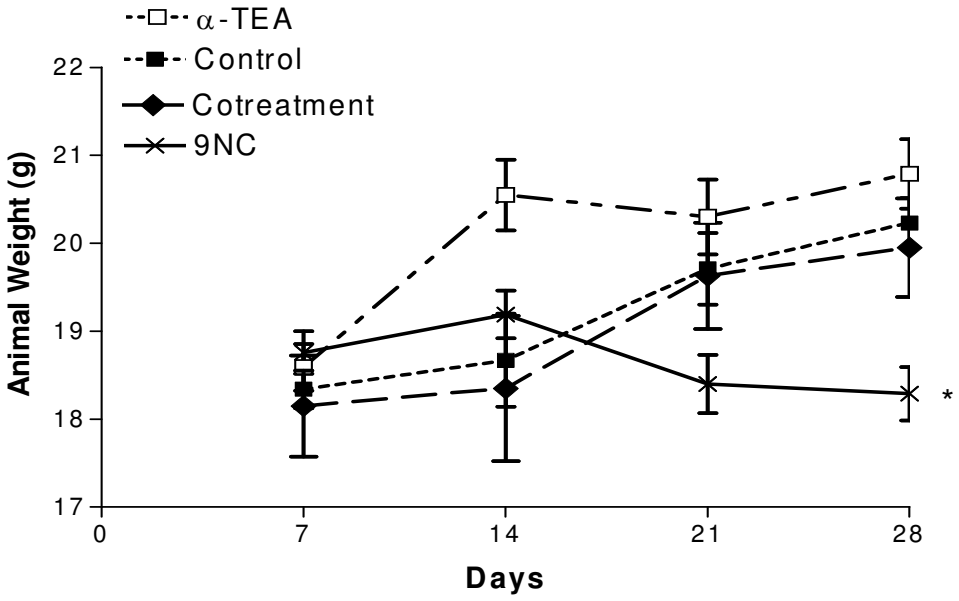


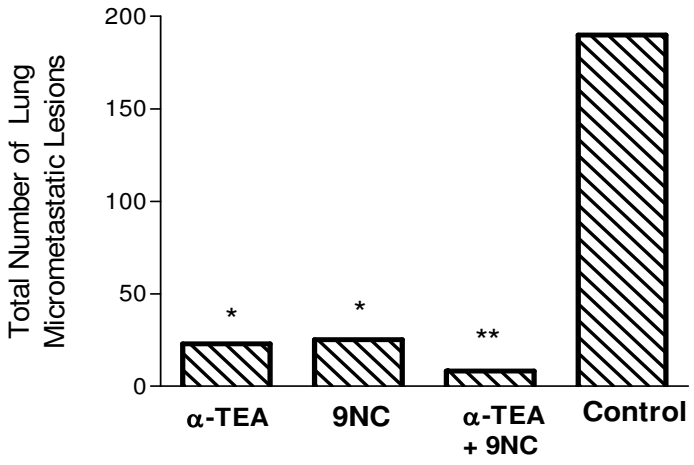
Figure 2.6: (A) Aerosolized α-TEA, 9NC, and combination treatments reduced tumor volume. Treatments were started 10 days after subcutaneous injection of $2x10^5$ 66cl-4-GFP cells/mouse and continued 7 days per week for 29 days. (B) Animal weight of the four treatment groups are measured over the duration of the study. Animal weights were measured every other day after the first week of the study
* significantly different from individual treatment groups; $P<0.05$

Table 2.1: The number of visible metastasis seen on the lungs and other locations were noted at the time of sacrifice

Treatments Delivered via aerosol	# of animals/group showing macroscopic lung metastasis [a]	Total # of macroscopic lung tumor foci [b]
Control	5/10	22
∝-TEA	6/10	21
9NC	7/10	14
∝-TEA + 9NC	4/10	10*

[a] Macroscopic metastatic lesions in all five lung lobes in each animal in all four treatment groups were counted visually at the time of sacrifice.
[b] Data presented represents the total number of visible lung macroscopic metastasis observed in all 10 mice in each group.
* Designates significant difference when compared to control.

A.



B.

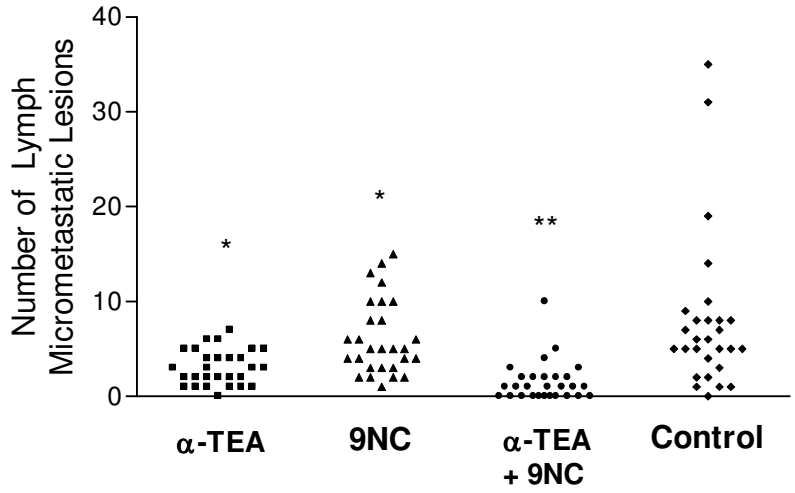


Figure 2.7: α –TEA, 9NC and combinations aerosol treatments exhibited a significant decrease in (A) lung and (B) lymph node metastasis.
* significantly different from control group; P<0.05
** significantly different from individual treatment group; P<0.05

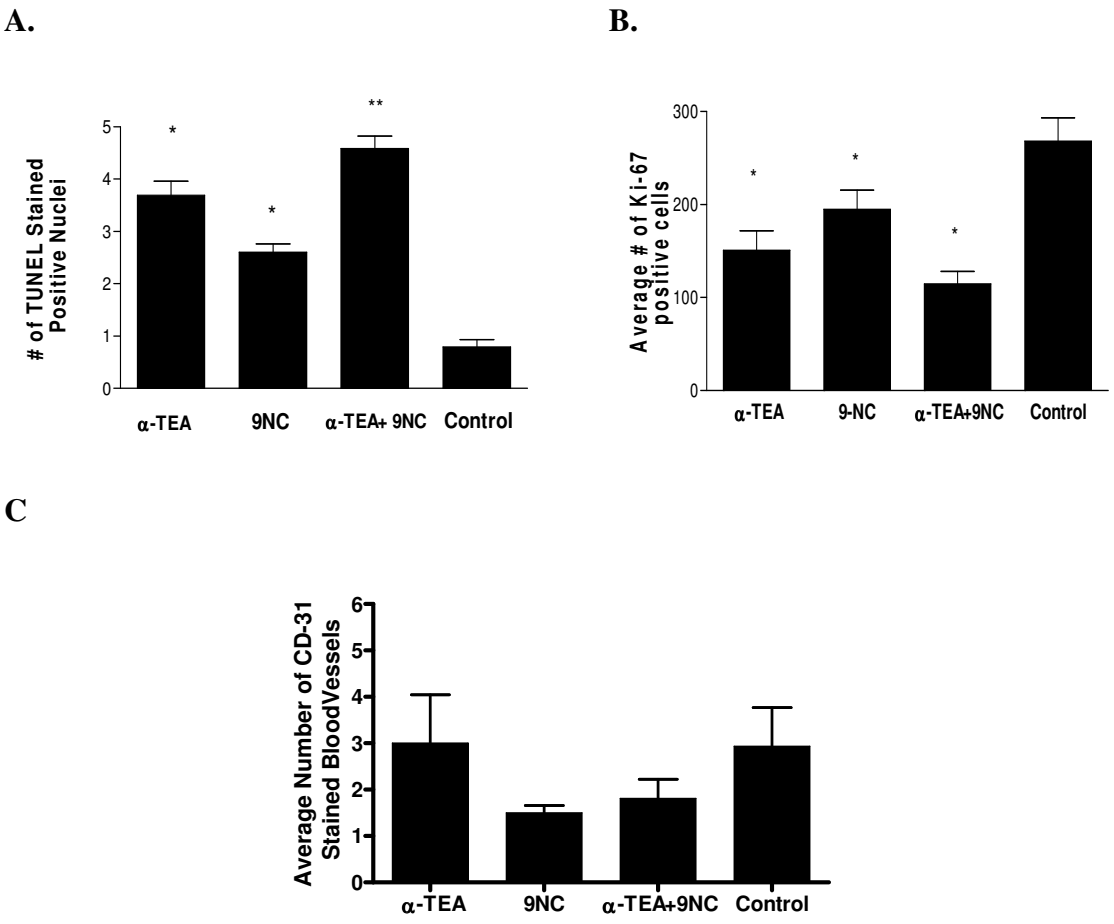


Figure 2.8: (A) α-TEA and 9NC induction of apoptosis in vivo was investigated using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. TUNEL-positive nuclei were counted at 400X magnification. All positive nuclei were counted in 16 separate fields on each slide. Ten separate tumors from each group were counted. (B) Both combination and individual treatments of α-TEA and 9NC, induce inhibition of 66cl-4-GFP cell proliferation *in vivo*. Cell proliferation was determined using 5 µm tumor sections derived from tumors from α -TEA , 9-NC, α -TEA and 9-NC and liposome aerosol control treatment groups. Proliferating cells were identified using Ki-67 antibody. 5 separate fields of each slide were scored for Ki-67 positive cells. Data are mean ± S.E. of all tumors in each group. Cells were counted with 400X magnification using a Zeiss ICM 405 fluorescent microscope under visual light with a Kodak digital imaging system. (C) α-TEA, 9NC and α-TEA+9NC treatment groups showed little effect on angiogenesis as determined by CD31 staining. Blood vessels were identified using the CD31 antibody where 5µm tumor sections were counted for CD31-positive staining.

* significantly different from control group; $P<0.05$

** significantly different from 9NC treatment group; $P<0.05$

Chapter 3: Study of a pro-apoptotic mechanism of action in 66cl4-GFP murine mammary cancer cells using novel vitamin E analog α-TEA

Abstract

Previous studies in our lab have shown that RRR- α-tocopherol succinate (VES) is a potent inducer or apoptosis in MDA-MB-435 human breast cancer cells. Since VES was able to restore the CD95(FAS) signaling pathway in these cells, the purpose of this study was to investigate the ability of α-TEA to induce apoptosis in 66cl-4-GFP murine mammary cancer cells, then to determine the level of involvement played by the CD95(Fas) signaling pathway in the induction of programmed cell death..

Data suggest that α-TEA induces apoptosis, at least partially, through the CD95 (Fas) pathway. Although the addition of Fas ligand was unable to activate apoptosis, data show that Fas still plays a vital role as after the addition of an anti-Fas neutralizing antibody, α-TEA induced apoptosis was significantly reduced. Although addition of Fas ligand had little effect, western immunoblot analysis show that α-TEA increased Fas ligand protein levels in a time dependent manner. α-TEA was also shown to perpetuate movement of Fas to the cell membrane, with the highest Fas protein concentration at the cell surface being seen at 6 hours following α-TEA treatment. Caspases 8, 9 and 3 appear to be downstream players but the level of their involvement may differ, based on the levels of subsequent cleavage. Mitochondrial involvement was highlighted by the

activation of Bax and truncation of Bid by α-TEA treatment. An alternative pathway involving ERK1/2, JNK and transcription factor c-Jun also appear to be involved as they were up-regulated in response to treatment with α-TEA. This was similar to the mechanism shown with VES and α-TEA in MDA-MB-435 human breast cancer cells. For the first time we have an insight into how α-TEA is able to induce apoptosis in 66cl-4-GFP murine mammary cells. This information will help future studies characterize the anti-apoptotic properties of this novel drug.

Introduction

Vitamin E is an umbrella term for a group of eight naturally occurring compounds that can be categorized as tocopherols or tocotrienols. Synthetic vitamin E which is made of a mixture of 8 sterioisomers along with succinate and acetate ester derivatives of both (RRR-) and synthetic (*all-rac* or dl) α-tocopherol, are also referred to as vitamin E (89)

The need for a non-toxic, non-hydrolizable chemotherapeutic agent led to the production of RRR-α-tocopherol (2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxy) acetic acid, α-TEA. Previous studies have shown the mechanisms of action of a structurally similar compound, RRR-α-tocopherol succinate (VES). VES was able to induce DNA synthesis arrest, differentiation and apoptosis in MDA-MB-435 cells. α-TEA activated programmed cell death via CD95 (Fas) signaling pathways via both mitochondrial dependent and independent pathways. α-TEA was determined to play an intricate role in JNK's phosphorylation of transcription factor c-Jun and Bax's ability to

translocate to the mitochondria causing mitochondrial permeability transition with eventual cytochrome-c leakage, caspase activation and then apoptosis (2,3,4,5,6,7). The rationale for this study was based on the results that were previously seen using α-TEA in vitro and in vivo, where primary tumor burden, lung and lymph nodes metastasis were inhibited (26,79,90)

Fas/APO-1/CD95 is a 36 kDa protein that is a member of the tumor necrosis factor (TNF) receptor superfamily This family of transmembrane receptors also includes TNF-α, and a variety of other cell surface receptors. Fas has been shown to have a pivotal role in the induction of apoptosis (91-94)

Briefly, Fas ligand (Fas-L) binds to membrane bound Fas inducing trimerization of the receptor. This activation causes the recruitment of FADD (Fas-associated death domain) an eventual interaction between the death domains of Fas and FADD. This death inducing signaling complex is now free to interact with downstream effector caspases. Procaspase 8 binds to the DISC leading to its activation. Once activated, caspase 8 cleaves other procaspases, in effect beginning a caspase cascade that ultimately leads to apoptosis. It has been shown that Fas signaling may be altered in several cancer cell lines (6,92,95,96)

MAPKs refer to a family of kinases that transduce a wide range of extracellular stimuli such as mitogenic growth factors, environmental stresses, and proapoptotic agents to the nucleus. The mechanism by which they achieve this is via sequential phosphorylation of

downstream players, allowing the regulation of proliferation, DNA synthesis arrest, differentiation, and apoptosis (97-100). To date there are at least three distinct MAP kinase signal transduction pathways in mammalian cells. These include the extracellular signal-regulated kinases, ERK 1/2 (also known as MAPKs), the c-JUN N-terminal kinases/stress-activated protein kinase (JNK/SAPK) and the p38 kinases.

In order to nullify apoptotic signals, many tumor cells, overexpress inhibitors of apoptosis proteins (IAP's) such as XIAP (X-linked inhibitor of apoptosis), c-Flip(L) and survivin. It is hoped that treatment with α-TEA will be able to down-regulate IAP's perhaps by inhibitors of IAPs, such as SMAC, and therefore allow the propagation of apoptosis (28, 36,101).

The data presented here shows that α-TEA induces apoptosis in 66cl-4-GFP mammary cancer cells not only in a concentration but also in a time dependent manner. The signaling pathway by which α-TEA is able to trigger apoptosis was shown to involve CD95(Fas) utilizing mitochondrial dependent, independent, and MAPK signaling. These findings provide a critical piece of the puzzle and help us gain a better understanding of the mechanism by which α-TEA is able to induce apoptosis.

Methods and Materials

Chemicals and Reagents

Treatments were conducted at various concentrations of α-TEA in a final concentration of 0.1% ethanol (8). Vehicle (VEH) treatments consisted of an equivalent amount of

sodium succinate for the highest amount of α-TEA used in the experiment in a final concentration of 0.1% ethanol. The following antibodies were used: PARP (sc-7150, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GAPDH (made in-house), Fas (sc-716, Santa Cruz), Fas-L (sc-834,Santa Cruz), Caspase-9 (sc-7558, Santa Cruz), Capase- 8 (sc-7890, Santa Cruz), Caspase-3 (sc-7148, Santa Cruz), Bax (sc-526, Santa Cruz), Bid (AF860, R&D Systems, Minneapolis, MN), JNK1 (sc-474, Santa Cruz), p-JNK (sc-6254, Santa Cruz), c-Jun (sc-1694, Santa Cruz), p-c-Jun (sc-822, Santa Cruz), DAXX (sc-7152, Santa Cruz), ERK1 (sc-94, Santa Cruz), p-ERK (sc-7383, Santa Cruz), Survivin (PSC-2237-C100, Axxora, San Diego, CA), c-Flip(L) (sc-5276, Santa Cruz), XIAP (#2042, Cell Signaling Technology, Beverly, MA), SMAC (sc-12683, Santa Cruz), Fas Ligand (FasL) membrane bound (#01-210, Upstate Biotechnology, Inc., Lake Placid, NY). DAXX siRNA(m) (sc-35177, Santa Cruz), siRNA silencer control was purchased from Ambion (Austin, TX). Myc-tagged survivin pcDNA3 plasmid, was a kind gift from Dr. J.Q. Chen (Department of Pathology, Molecular Oncology, and Drug Discovery Center & Research Institute, Tampa, Florida), Wild type HA-tagged c-Flip(L) was a provided by Dr. John. Reed (The Burnham Inst. La Jolla, CA), Dominant negative (DN) mutant of JNK1 was a kind gift from Dr Roger Davis (Howard Hughes Medical Institute, University of Massachusetts, Boston, MA). DN-c-Jun: of Dr. Michael J. Birrer (Molecular Biology Division, National Institutes of Health, National Cancer Institute, Rockville, MD), DN-Daxx (DAXX-c): Dr. David Baltimore(Department of Biology, California Institute of Technology, Pasadena, CA). Empty pcDNA vector was purchased from Invitrogen (Carlsbad, CA).

Cell Lines and Culture Conditions.

Murine Mammary Tumor Cell Line.

66cl-4 cells were derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone (33, 80). Previously these cells were stably transfected with the enhanced green fluorescent protein and selected for a high degree of fluorescence by Dr. L-Z Sun (UT Health Science Center, San Antonio, TX). 66cl-4-GFP cells have been shown to be highly metastatic, with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing microscopic metastases detectable with fluorescent microscopy in the lungs 26 days following s.c. injection of 2 X 10^5 tumor cells into the inguinal area (26). 66cl-4-GFP cells were maintained as monolayer cultures in McCoy's media (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 100 µg/ml streptomycin, 100 IU/ml penicillin, 1 X (vol/vol) non-essential amino acids, 1X (vol/vol) MEM vitamins, 1.5 mM sodium pyruvate, and 50 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO). Cell culture experiments involved culturing the cells in this same McCoy's supplemented media except that FBS content was reduced to 5%. The human T cell Leukemia cells, Jurkat, clone E6-1, were purchased from the American Type Culture Collection and cultured according to ATCC's instructions. Cultures were routinely examined to verify absence of mycoplasma contamination.

Analysis of Apoptosis Via Morphological Evaluation of DAPI-Stained Nuclei.

Apoptosis was determined using previously published procedures (66,87). Cells were plated at 1 x10^5 cells/well in 12-well plates were cultured overnight to permit attachment. The cells were then treated for 3 days after which they were collected and floating cells plus scraped-released adherent cells were pelleted by centrifugation for 5 min at 350 X *g*, washed one time with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCL, 10.4 mM Na_2HPO_4, 10.5 mM KH_2PO_4; pH 7.2), and stained with 2 µg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim, Indianapolis,

IN) in 100% methanol for 15 min at 37˚C. Cells were viewed at 400X magnification with a Zeiss ICM 405 fluorescent microscope using a 487701 filter. Cells in which the nucleus contained condensed chromatin, or cells exhibiting fragmented nuclei, blebbing, were scored as apoptotic. Data are reported as percentage of apoptotic cells per cell population (i.e. number apoptotic cells/total number of cells counted). Three different microscopic fields were examined and 200 cells counted at each location for a minimum of 600 cells counted per slide. Apoptotic data are presented as mean ± S.D. for three independently conducted experiments.

Western Immunoblot Analysis

66cl-4-GFP cells were plated at a density of 1.5 x 10^5 cells/ml in T-25 flasks (10 ml/flask) and allowed to adhere overnight. Next, the media were removed and replaced with experimental media containing treatments of α-TEA (20µg/ml) and VEH. Cells were treated with 20µg/ml α-TEA for either 1,2,3,4,6,15 or 24 hours and then collected. by scraping to combine floating and adherent cells, and then pelleted by centrifugation at 350 g. To determine presence and levels of protein within the cell, whole cell lysates were prepared as previously described (66,87). Cells were then washed in PBS to remove any remaining media and lysed with lysis buffer (1x PBS, 1% NP40, 0.5% sodium deoxyxholate, and 0.1% SDS) added to 1µg/ml aprotinin and leupeptin, 1mM DTT and finally 2 mM sodium orthovadate. The lysis mixture is incubated on ice for 30 minutes. Lysates are then centrifuged at 15,000 x g for 10 minutes at 4 0 C and then protein concentrations determined following the Bio Rad Dye Binding protein assay. 50µg of protein was used per lane and either run on a 10%, 12% or 15% SDS-page gel

under reducing conditions (79,90). PARP cleavage by caspase 3 serves as an early indicator of apoptosis (88).

Primary antibodies used were previously listed and are used in conjunction with peroxidase-conjugated goat anti-rabbit, goat-anti-mouse (Jackson Immunoresearch Laboratory, West Grove, PA) at a 1:1000-1:5000 dilution. Detection was performed using ECL (Pierce, Rockford, IL) and densitometry determined using Scion Image Software (Scion Corporation, Frederick, MD). To determine equal lane loading in each experiment, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in our lab) was used.

Fas Ligand Activation Assay

Cells were plated at $1.5x10^5$ cells/well in 12 well plates and then treated with Fas ligand (01-210, Upstate Biotechnology, Inc., Lake Placid, NY) at a concentration of 1:1000 through 1:5000 for 24 hours. Human Jurkat cells were plated at the same concentration and used as a positive control. Pretreatment experiment used α-TEA at 2.5, 5 and 10μg/ml for 6 hours prior to the addition of Fas ligand. Cells were then collected and DAPI analysis performed to determine percentage of apoptotic positive cells.

Fas Neutralization Assay

Cells were treated for 72 hours with either 2.5, 5 or 10µg/ml α-TEA plus 0.5µg/ml of Fas neutralizing antibody (ZB4; Immunotech, Westbrook, ME). Cells positive for apoptosis were then determined via DAPI staining.

Bax Activation Assay.

In order to detect the active, conformationally changed fraction of Bax, the protocol decribed by Yamaguichi et al (105) paper was followed. Cells were collected and pelleted and then lysed with CHAPS lysis buffer [10 mM HEPES (pH 7.5), 150 mM NaCl, 1% CHAPS] in the presence of protease inhibitors for 30 mins. A total of 500 µg of protein on 0.05 ml of CHAPS lysis buffer was incubated overnight at 4^{o} C with 2 µg of anti-Bax 6A7 monoclonal antibody. 20µl of Protein G Agarose beads was then added to the lysis mixture and was then incubated at 4^{o} C for 2 hours, followed by three washes in CHAPS lysis buffer. The beads were then boiled in Laemmli buffer and the presence of immunoprecipitated, conformationally changed Bax protein was detected via Western Immunoblotting using anti-Bax polyclonal antibody (sc-526).

Caspase Inhibitors

66cl-4-GFP cells were plated at a concentration of $1.5x10^{5}$ cells/well in 12 well plates and then treated with one of the following caspase inhibitors: Caspase-8 inhibitor (Z-IETD-FMK), caspase-3 inhibitor (Z-DEVD-FMK) and caspase-9 inhibitor (Z-VAD-FMK) were all purchased from BioVision Research Products (Palo Alto, CA), at 2µM for 2 hours.

They were then treated with 20µg/ml α-TEA for two days. To determine the percentage of cells undergoing apoptois, DAPI analysis was used.

JNK Inhibitor

66cl-4-GFP cells were plated at $1.5x10^5$ cells/well in 12 well plates and then treated with 40 nM of JNK inhibitor II (Calbiochem-Novabiochem Corp. San Diego, CA), or DMSO vehicle for 2 hours prior to treatment with α-TEA (10µg/ml or 20µg/ml) for 48 hours. To determine the inhibition of JNK activity, Western Immunoblot for JNK and p-c-Jun was performed.

Mitochondrial Fractionation

Mitochondrial enriched fractions were isolated using the Biovision mitochondrial/cytosol fractionation kit (BioVision Research Products, Mountain View, CA). Briefly treated cells were collected and washed with 10mls of ice cold PBS and then centrifuged at 600xg for 5 mins at 4°C. Supernatant was removed and resuspended with 1.0ml of 1X cytosol extraction buffer mix (1xPBS) containing 1mM DTT and protease inhibitors (1µg/ml aprotinin and leupeptin, 1mM DTT and finally 2 mM sodium orthovadate). Incubate on ice for 10 mins. Cells are then homogenized with 30-50 passes through at 26 gauge needle. Homogenates were then centrifuged at 700xg for 10 mins at 4°C. The supernatant was collected and transferred to a fresh 1.5ml tube and centrifuged at 10,000xg for 30 mins at 4°C. The collected supernatant contained the cytosolic fraction, and the pellet the mitochondrial fraction. The pellet was then lysed in 100µl of mitochondrial extraction buffer mix (1xPBS) containing 1mM DTT and protease

inhibitors (1µg/ml aprotinin and leupeptin, 1mM DTT and finally 2 mM sodium orthovadate). Protein concentration was then determined via Bio Rad Dye Binding protein assay. 50µg of protein was used per lane and either run on a 12% or 15% SDS-page gel under reducing conditions.

Functional knock down of DAXX

In order to inactivate DAXX, siRNA/siPORT reagent was prepared by mixing 0.2ml serum free media and 3.6µl siPORT NeoFx reagent. This mixture was then incubated for 10 mins with siRNA to DAXX, or a silent negative control. The final concentration of siRNA was 30nM in a total volume of 0.6ml. 66cl-4-GFP cells were collected via trypsinization and resuspended in the serum free media at $3.75x10^5$ cells/ml. Using 12 well plates, 0.2ml of the reagent mix along with 0.4ml of cell suspension were added to each well and allowed to incubate overnight. The next day the serum free media was replaced with normal culture media and cells were incubated for a following 24 hours. Cells were then treated with α-TEA (20µg/ml) or vehicle for 24 hours. The two treatment groups were divided to be analyzed via DAPI staining or Western Immunoblot.

Transient Transfection

66cl-4-GFP cells were plated at a concentration of $1.5x10^5$ cells/ml in 12 well plates and then allowed to adhere overnight. Prior to transfection with DN-JNK1, DN-c-Jun, Myc-tagged survivin, HA-tagged c-Flip(L), DN-DAXX (DAXX-c) or vector controls, cells were washed with serum free media. Briefly, 0.7µg of DNA was added to 50µl serum

free media containing 4µl of Plus (Invitrogen, Carlsbad, CA) reagent which was then incubated for 15 mins (mix 1). 2µl of LipofectAMINE reagent was added to 50µl serum free media and then combined with mix 1and incubated for a futher 15 mins. Cell were then allowed to incubate for 8 hours after which the media was replaced with normal culture media overnight. Cells were then treated with α-TEA (20µg/ml) for 24 hours. Success of transfection was determined via western immunoblot.

Statistical Analysis of Data

Statistical analysis of the data was performed using Prism software version 4.0 (Graphpad Prism, San Diego, CA) were a level of $P<0.05$ was considered to be statistically significant. Apoptosis data was analyzed using a one way ANOVA followed by a Tukeys's post-hoc test.

Results

α-TEA Induced Apoptosis in a Concentration and Time Dependent Manner in 66cl-4-GFP Murine Mammary Cancer Cells.

66cl-4-GFP cells treated with 5,10 and 20µg/ml α-TEA for 48 hours displayed a concentration dependent induction of apoptosis. Level of apoptosis induced by 5,10 and 20µg/ml of α-TEA was significantly increased in comparison to the vehicle control (Fig 3.1A). α-TEA was also able to induce apoptosis in a time dependent fashion as was seen via cleavage of the apoptotic marker protein PARP, and continued through the 48hr treatment period. The 84-kDa cleavage fragment was evident starting at 15 hours in comparison to the untreated vehicle control where no cleavage was seen (Fig 3.1B).

CD 95(FAS) Signaling Pathway Appears to be Involved in α-TEA Induced Apoptosis.

To determine if Fas plays a role in the induction of apoptosis in 66cl-4-GFP cells using α-TEA, we first examined the effect of addition of membrane bound Fas ligand to plated cells. What we discovered was that adding Fas ligand at concentrations ranging from 1:1000 through 1:5000 had little or no ability to induce apoptosis. Levels of apoptosis in positive control Jurkat cells was significantly enhanced at both concentrations of FasL (Fig. 3.2A). Previous studies in our lab have shown that a similar compound to α-TEA, VES, can reactivate Fas signaling by pretreating the cells prior to stimulation with Fas ligand (26). This study failed to show an increase in apoptotic levels after a 6 hour pretreatment (Fig. 3.2B). To further investigate if Fas was involved, we tackled the question from a slightly different angle. We next used Fas neutralizing antibody in conjunction with α-TEA treatments of 2.5,5,10 and 20μg/ml and measured the neutralizing antibody effects on apoptosis via DAPI staining. Inhibition of Fas, significantly decreased α-TEA's ability to induce apoptosis at increasing concentrations, when compared to the irrelevant antibody control. (Fig. 3.3A). Summarizing these findings we were able to conclude that Fas is involved in α-TEA induction of apoptosis in this murine cell line.

α-TEA Increased Levels of Membrane Bound Fas as well as Fas Ligand in a time dependent manner.

Western Immunoblot analysis showed that α-TEA at a concentration of 20μg/ml increased membrane bound Fas at time points 3 to 24 hours. Levels of total Fas in cell extract did not change with α-TEA treatment. The peak level of membrane bound Fas was seen at 6 hours and continued to decline through 24 hours (Fig 3.3B). Fas ligand levels showed a slight increase from 3 to 24 hours, but was not significantly different from the control (Fig. 3.3B). This may suggest that α-TEA acts to help Fas translocate from the cell cytoplasm to the membrane of the cell as opposed to increasing overall cellular Fas or Fas ligand levels.

α-TEA utilizes a caspase dependent signaling pathway for induction of apoptosis in 66cl-4-GFP cells.

In order to determine the involvement of caspase 3, 8 and 9 in α-TEA induced apoptosis, 66cl-4-GFP cells were pre-treated with caspase specific inhibitors followed by α-TEA treatment and then levels of apoptosis were measured. Addition of inhibitors specific to caspases 3,8 and 9 significantly reduced apoptosis in α-TEA treated cells when compared to cells treated with α-TEA alone (Fig.3.4A). In order to determine the time at which caspases are activated, cells were treated with α-TEA (20μg/ml) for 3,6,15 and 24 hours and then western immunoblot was used to determine presence of caspase cleavage. Caspase 8 cleavage was first seen at 3 hours, peaked at 15 hours and then decreased over 24 hours (Fig 3.4B, panel 1). Cleavage bands for caspases 9 and 3 were first seen at 15

hours and continued through 24 hours (Fig. 3.4B, panels 2 and 3). GAPDH served as lane load controls.

These data suggest that α-TEA induced apoptosis is caspase dependent, specifically working through caspases 3, 8 and 9. Based on data generated in this chapter, a proposed signaling pathway is depicted in Figure 3.5.

Increase in tBid and conformationally active Bax protein levels, suggest that α-TEA induces apoptosis through a mitochondrial dependent pathway.

To further understand the apoptotic signaling pathway downstream of Fas, additional studies were conducted. Although the levels of Bax in the whole cell did not change in response to treatment, the role of Bax in α-TEA induced apoptosis is supported by an increase in the conformationally changed form of Bax (Fig 3.6, panel 1). The importance of the mitochondria is further highlighted by the presence of the truncated form of Bid (tBid) (Fig 3.6, panel 2). Both tBid and the active form of Bax are detected at 15 hours after treatment with α-TEA. (Fig. 3.6, panels 2 and 3)

MAPK pathway players JNK1 and ERK1/2 are activated by α-TEA In 66cl-4-GFP Cells.

To address a possible role for JNK,member of the MAPK pathway, we first investigated the effect of a JNK inhibitor (JNK inhibitor II) on the ability of α-TEA (20μg/ml) to induce apoptosis. JNK plays a significant role in that a decrease of approximately 20% in apoptotic cells is seen in cells pre-treated with the inhibitor + α-TEA, as well as reduced cleavage of PARP. (Fig. 3.7 A, B). Knowing that JNK is integral to α-TEA induced

apoptosis, we wanted to determine at what time α-TEA actives JNK, and the effect on players downstream, namely c-Jun. Cells were again treated at a concentration of 20μg/ml for 3,6,15 and 24 hours and then Western Immunoblot was performed. The active form of JNK, p-JNK was detected starting at 6 hours, continued to increase and peaked at 24 hours (Fig 3.7C, panel 2). Transcription factor c-Jun is phosphorylated starting at 3 hours and continues to increase through 24 hours. Since c-Jun is activated by JNK, and is downstream, one would expect it to be activated around the same time point (6 hrs). This may suggest another player is activating c-Jun at an earlier time point (Fig.3.7C, panel 2). Investigating how much control JNK has in the activation of c-Jun, we next cultured the cells with JNK inhibitor II in the presence and absence of α-TEA. As expected the levels of apoptosis after treatment with α-TEA were decreased in cells that expressed the dominant negative version of JNK(Fig 3.7D, panel 1). It was also noted that levels of phosphorylated c-Jun (pc-Jun) were also decreased. (Fig. 3.7D, panel 2) This suggests that JNK plays a significant role in the activation of c-Jun and that JNK/c-Jun are involved in α-TEA induced apoptosis. Together the importance of JNK and c-Jun is evident from the overall decrease in apoptotic positive cells after culturing the cells with both dominant negative JNK and c-Jun plus α-TEA. (Fig. 3.7D). α-TEA treatment caused in increase in the active form of ERK1/2, starting at 1 hour, peaking at 3 hrs and remaining high at 6 hrs of treatment (Fig 3.8). Levels of pERK1/2 began to decline at 4 hours (Fig. 3.8)

Daxx does not play a central role in α-TEA induced apoptosis

Daxx (death associated protein 6) is a novel death domain-containing protein that is ubiquitously expressed, originally identified through a yeast two-hybrid screen as an interactor with the cytoplasmic domain of Fas (53). Studies have shown that DAXX was able to enhance Fas-mediated apoptosis and activate the JNK pathway (53). The particular role that DAXX plays still remains controversial, as there has been convincing evidence showing that it can either promote or hinder apoptosis (53,125-129). Studies conducted here aim to elucidate if DAXX plays a role in α-TEA induced apoptosis, and if it does, via what signaling pathway.

To determine if DAXX was involved in the induction of apoptosis, cells containing dominant negative Daxx (Daxx C), α-TEA, negative siRNA and DAXX siRNA were treated with α-TEA (20μg/ml) for 48 hours and then evaluated for apoptosis using DAPI staining. C-DAXX or DAXX siRNA treatments did not inhibit α-TEA induced apoptosis (Fig 3.9A). Western immunoblot was used to confirm reduced levels of DAXX protein is cells treated with DAXX siRNA (Fig. 3.9B).

Cells were treated with α-TEA (20μg/ml) with or without C-DAXX for 24 hours and then levels of DAXX p-JNK1, pc-Jun and PARP cleavage were determined. Levels of p-JNK1, pc-Jun or PARP cleavage were not substantially different from vector control α-TEA treated cells (Fig 3.9C). Together this data provides convincing evidence that DAXX plays no significant role in α-TEA induced apoptosis.

α-TEA inhibits pro-survival proteins

Pro-survival proteins survivin and, c-Flip(L) are potent regulators in ensuring tumor cell survival. To investigate their importance in α-TEA induced apoptosis, 66cl-4-GFP cells overexpressing pcDNA survivin and pcDNA c-Flip(L) were treated with α-TEA (20μg/ml) for 48 hours and evaluated for apoptosis via DAPI staining. α-TEA induced apoptosis was significantly reduced in cells cultured with both pcDNA survivin and pcDNA c-Flip(L) (Fig 3.10A) Apoptotic levels were significantly reduced ($P < 0.001$, $P < 0.001$) in cells expressing either c-Flip(L) (72%) or survivin (69%) when compared to α-TEA treated control vector (Fig 3.10A). Western Immunoblot was used to confirm the presence of elevated c-Flip(L) and survivin (Fig.3.10B). Western Immunoblot showed that α-TEA (20μg/ml) treatment for 3,6,15 and 24 hrs reduced cellular levels of c-Flip(L), survivin and XIAP over a 24 hour period. Furthermore, mitochondrial SMAC levels decreased when examined in a mitochondrial enriched fraction, suggesting its movement into the cytosol where it continues to function in a pro-apoptotic capacity (Fig. 3.10C)

Pre-treating 66cl-4-GFP cells with α-TEA restores sensitivity to TGF-β.

TGF-β is known to play a crucial role in the induction of apoptosis in many different types of cancer cells. With this in mind the aim of this study was to determine the role, if any that TGF-β1 may play in α-TEA induced apoptosis. To first address this question 66cl-4-GFP cells were treated with TGF-β and the effect on DNA synthesis was studied (Fig 3.11A). The data showed that that TGF-β, did not have any significant ability of induce DNA synthesis arrest in this cell line. TGF-β induced DNA synthesis arrest in

positive control MCF-10A cells (Fig 3.11A). To investigate the possibility that α-TEA may be able to restore sensitivity to TGF-β, the cells were pretreated with 5 and 20 μg/ml α-TEA for 12 hours and then treated with different concentrations of TGF-β. All concentrations of TGF-β induced significant levels of DNA synthesis arrest in this cell line following pretreatment with α-TEA (Fig 3.11B). Western blot analysis showed that α-TEA(20μg/ml) was able to increase TGFβR II starting at 3 hours, reaching peak levels at 6 hours, with decreased levels at 15 hours and non-detectable levels at 24 hrs (Fig 3.11C). Together these data suggest that TGFβ plays an important role in α-TEA induced apoptosis in 66cl-4-GFP cells. Further studies are warranted.

Discussion

Studies reported here provide an invaluable mechanistic insight into how α-TEA induces programmed cell death (apoptosis) in 66cl-4-GFP murine mammary cancer cells. Treatments were not only able to induce cell death in a time dependent, but also a concentration dependent manner. Data suggests that during the timeframe of apoptotic stimulation, Fas plays a major role in the transmission of death signals, followed by a wide range of intermediate players , including mitochondrial, and caspase associated pathways.

α-TEA treatment of murine mammary cancer cells increased levels of membrane bound Fas suggesting that this novel vitamin E derivative is facilitating the translocation of Fas from the cell cytoplasm to the cell membrane. The exact mechanism by which α-TEA modulates this process is not currently known, and is an area of interest that needs further

investigation. The involvement of caspases was then investigated specifically by looking at caspase 8, 9 and 3. Based on the literature there are two well reported Fas signaling pathways (107). Type-1 apoptosis involves high levels of caspase 8 cleavage followed by direct activation of caspase 3 leading to rapid cell death. This mitochondrial independent pathway is classically faster as it requires fewer intermediate players. In Type-II apoptosis, caspase 8 cleavage is not as prominent, rather its slow activation mediates downstream apoptotic events primarily through truncation of Bid to t-Bid followed by the induction of mitochondrial damage. Mitochondrial permeability transition is regulated via truncation of Bid and translocation of Bax from the mitochondria to the cytosol. This permeability change allows cytochrome-c leakage and eventual activation of caspase 9 and 3 leading to cell death. It has been shown that Fas can initiate an apoptotic pathway independent of key players such as FADD and caspase 8 (53) suggesting that Fas mediated apoptosis is a lot more complicated than previously imagined. Western immunoblot showed that there was caspase 8 cleavage, but at much lower levels than caspases 9 or 3, but did confirm that the primary apoptotic pathway utilizes the mitochondria, as was seen with an increase in the active from of Bax and the truncation of Bid. This would suggest that the primary pathway that α-TEA is able to induce apoptosis is via the Type-II signaling pathway.

Investigation of a proposed caspase independent death pathway revealed that α-TEA increased protein levels of JNK and its downstream target c-Jun. To determine if JNK is an essential player in the induction of apoptosis, JNK was chemically inhibited as well as

a double negative mutant of JNK used. Results indicate that JNK plays a pivotal role in α-TEA induced apoptosis as there was a significant decrease in apoptosis when JNK was inhibited.

The exact role of ERK1/2 in α-TEA treated cells has not been fully explored. α-TEA increased the levels of phosphorylated ERK starting at 1hour, peaking at 3 hours and slowly declining by 4 though 6 hours. This appears to be different to the majority of other ERK stimulus that are shown to be involved in cellular differentiation and proliferation, but does leave open the possibility that pERK1/2 is involved in cell death signaling (130).

In an effort to categorize the importance of other Fas associated proteins, namely DAXX, we used siRNA to DAXX as well as a double negative construct to determined if there was any significant effect on apoptotic levels. Daxx has been widely reported to be a pro-apoptotic protein signaling through direct interaction with Fas and downstream players such as JNK, and ASK-1 (53. 99-102). Data suggests that α-TEA signals apoptosis via a DAXX independent mechanism. Use of DN-DAXX as well as siRNA to DAXX had little or no effect on the ability of α-TEA to induce apoptosis.

The ability of α-TEA to induce apoptosis in 66cl-4-GFP cells has been shown to rely heavily on Fas activation, acting through a mitochondria dependent pathway, but it was of importance to determine the effect of α-TEA on inhibitor to apoptosis proteins (IAP's).

The IAP's that are the focus of this study were, caspase 8 (FLICE) inhibitor, c-Flip(L), Survivin (inhibits the proteolytic activity of caspase 3), and XIAP (inhibitor of caspase 9 and 3). The results show that overexpressing survivin and c-Flip(L) greatly hinder the ability of α-TEA to cause apoptosis in this cell line. Western immunoblot shows that reduced protein levels of these IAP's in α-TEA treated cells are detected over a 24 hour period. As well as reducing the ability of proteins that aim to inhibit apoptosis, treatment was successful at promoting the movement of an IAP inhibitor, SMAC from the mitochondria to the cytoplasm where it acts to maintain caspase activity.

Taken together, these results suggest that inhibitory proteins play a significant role in prevention of apoptosis. Knowing that elevated c-Flip(L), XIAP and survivin levels appear to confer resistance to apoptosis and promote tumorigenicity, interference with their induction by α-TEA may provide a novel therapeutic approach in facilitating the elimination of tumor cells.

Lastly, we investigated the role that TGF-β may play in α-TEA induced apoptosis. Pretreatment with α-TEA for 12 hours was able to resensitize the cell line to TGF-β activity as was see in the ability to induce DNA synthesis arrest. Western immunoblot analysis showed that TGF-β-receptor protein levels were increased at an early time point, 3 and 6 hours after treatment of α-TEA (20μg/ml). These data suggest that α-TEA is an effective agent in reestablishing TGF-β sensitivity in 66cl-4-GFP murine mammary cancer cells.

In summary, these findings suggest that a novel vitamin E analog, α-TEA is able to induce apoptosis in 66cl-4-GFP murine mammary cancer cells by activation of Fas and works through primarily a caspase dependent Type-II mechanism. A proposed signaling pathway is depicted in Fig 3.12

We determined that key pro-death players in this process include activation of effector caspases 9 and 3 as well as mitochondrial associated Bid and Bax. Early activation of ERK1/2 as well as the phosphorylation of JNK target c-Jun, in conjunction with downregulation of pro-survival signals all contribute to this unique multidirectional induction of apoptosis

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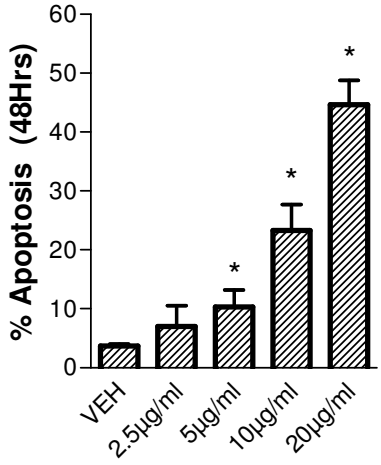
A



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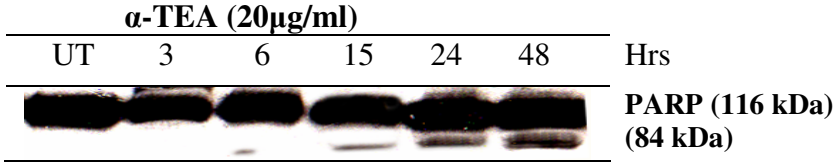


Figure 3.1 α-TEA induces apoptosis in 66cl-4-GFP murine mammary cancer cells in a time and concentration dependent manner. (A) Cells were treated with 2.5,5,10 or 20 µg/ml for 2 days and then evaluated for presence of apoptotic cells via DAPI staining. (B) Cell lysates for 66cl-4-GFP treated with 20µg/ml for 3,6,15,24 and hours were analyzed via western immunoblot for cleavage product (84kDa) of caspase 3 substrate PARP. (A) Data are depicted as the mean ± S.D. of three independent experiments were $*P < 0.05$. when compared to vehicle control (B) is representative of three different experiments.

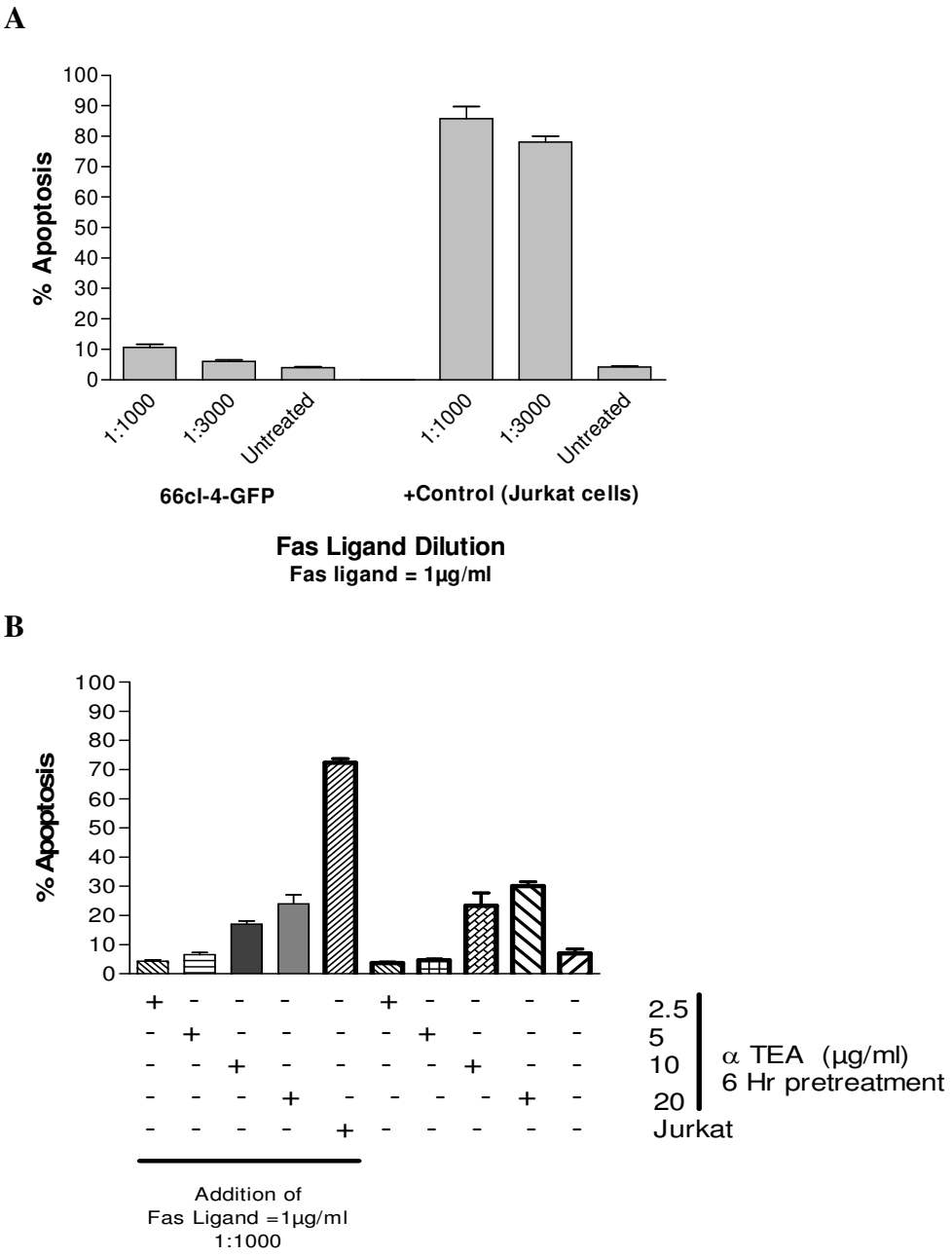
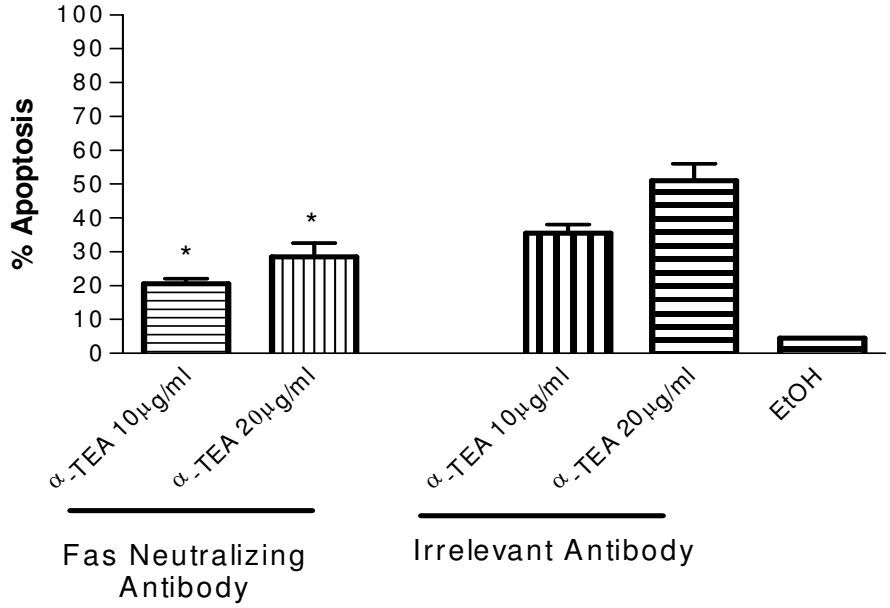


Figure 3.2. Addition of Fas ligand had no clear effect on apoptosis. 66cl-4-GFP cells treated with Fas ligand did not show any significant increase in apoptotic cells as determined by DAPI staining. (B) Pretreatment of cells with 2.5, 5 or 10µg/ml for 6 hours did not sensitize cells to Fas ligand induced apoptosis. (A and B) data are depicted as the mean ± S.D. of three independent experiments.

A.



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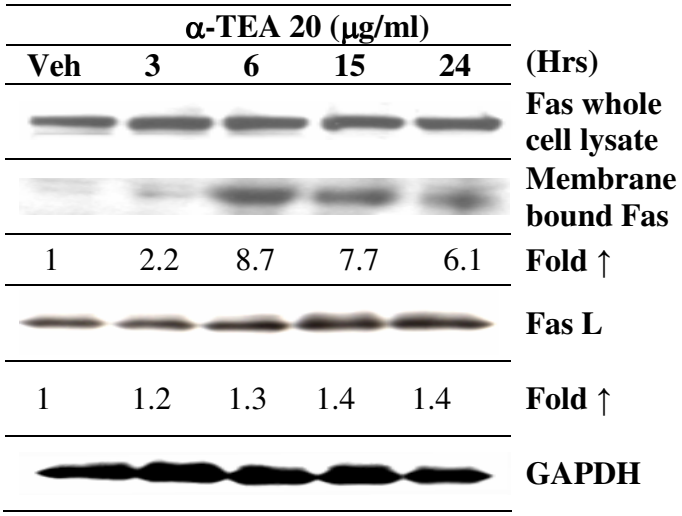


Figure 3.3. Fas plays an integral role in α-TEA induced apoptosis in 66cl-4-GFP cells. (A) Addition of an anti-Fas neutralizing antibody (0.5μg/ml) for 2 hours prior to treatment with 20μg/ml for 2 days, showed a significant decrease in apoptotic cells as determined via DAPI staining. (B) α-TEA treatment increases protein levels of membrane bound Fas and to a lesser extent Fas ligand. Cells treated with 20μg/ml for 3,6,15 and 24 hours were analyzed via western immunoblot to detect levels of membrane bound Fas and Fas ligand. Data is representative of at least 2 experiments.
Data are depicted as the mean ± S.D. of three independent experiments were $*P < 0.05$. when compared to treated cells without neutralizing antibody.

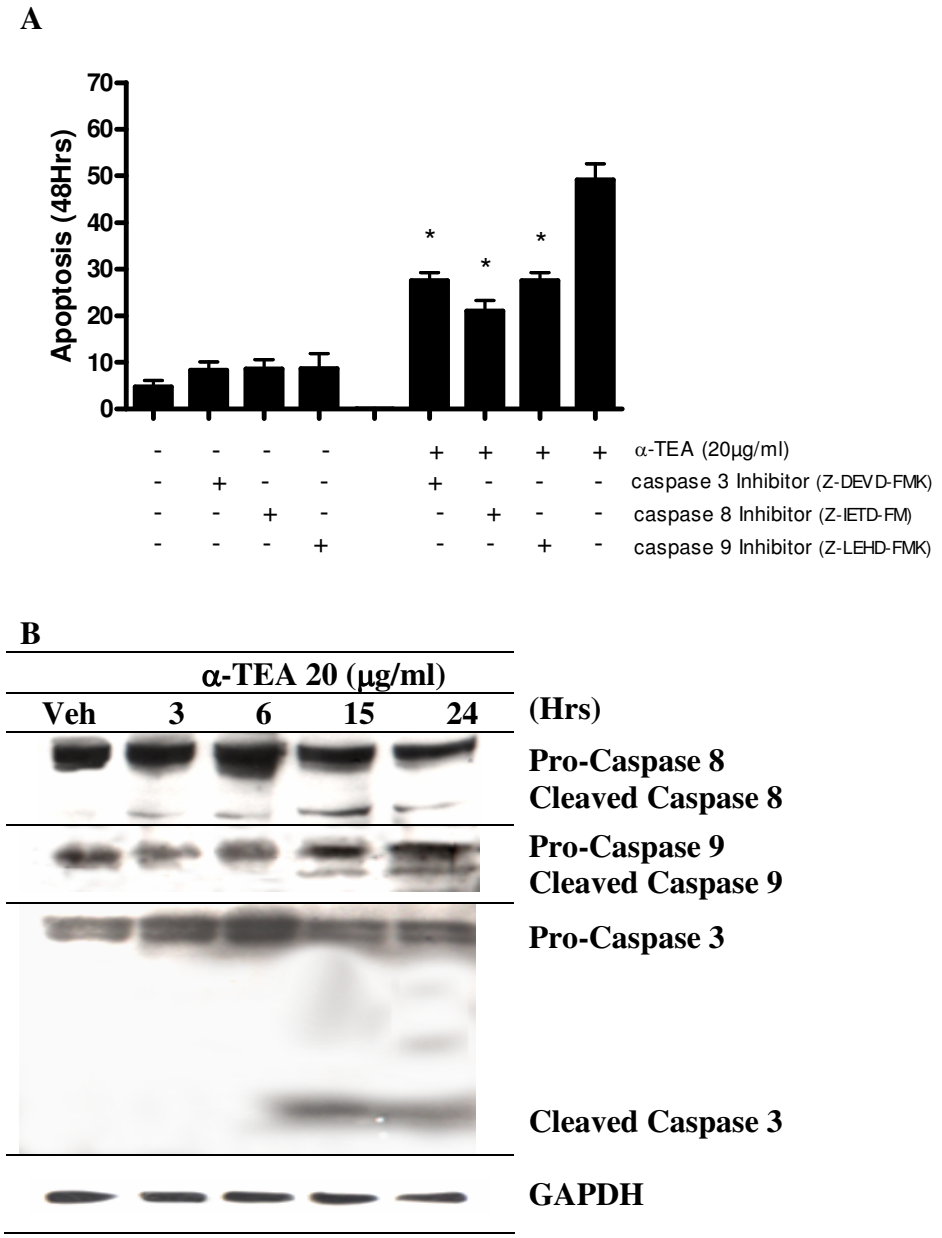


Figure 3.4 α-TEA induced apoptosis is dependent of caspase 3,8 and 9. (A) Cells were pretreated with caspase 3, 8 and 9 inhibitors (Z-DEVD-FMK, Z-IETD-FM and Z-LEHD-FMK, 2μM) respectively for 2 hours prior to treatment with α-TEA (20μg/ml). DAPI staining was used to determine the percentage of apoptotic positive cells. Data represents three independent experiments. * = $P < 0.05$ when compared to α-TEA (20μg/ml) alone. (B) Cells were treated with α-TEA (20μg/ml) for 3,6,15 or 24 hours and western immunoblot used to detect the effect of treatment on caspase 3,8, and 9 cleavage, with GAPDH used as a loading control. Data was representative of three independent experiments.

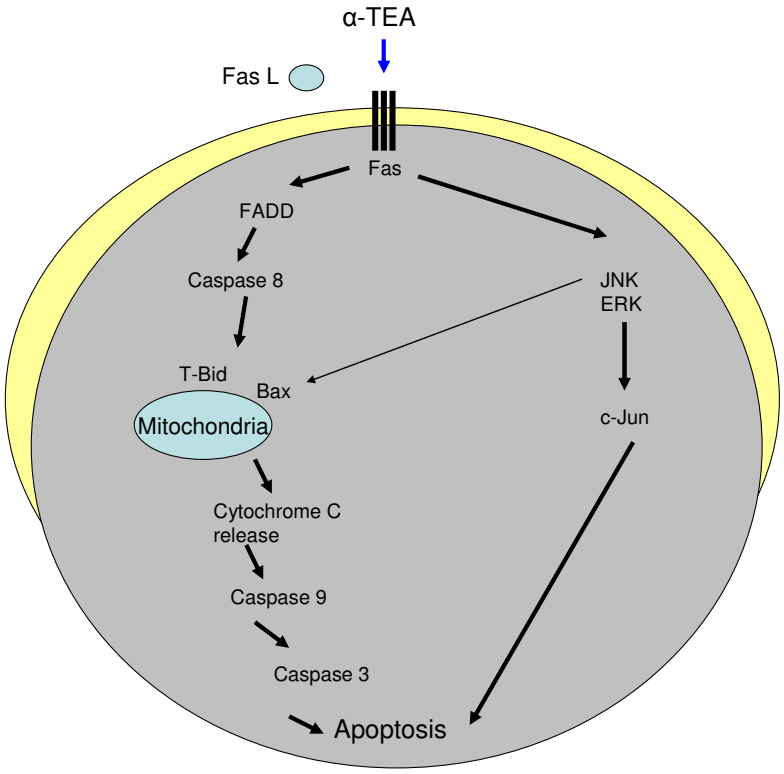


Figure 3.5. Proposed signaling mechanism of α-TEA induced apoptosis involves signaling through Fas/Fas L causing activation of pro-caspase 8 and eventual mitochondrial permeability transition via association of Bax and Bid. Cytochrome c release follows and subsequent activation of effector caspases 9 and 3 eventually causing cell death. A mitochondrial independent pathway is thought to be mediated through JNK, ERK and c-Jun

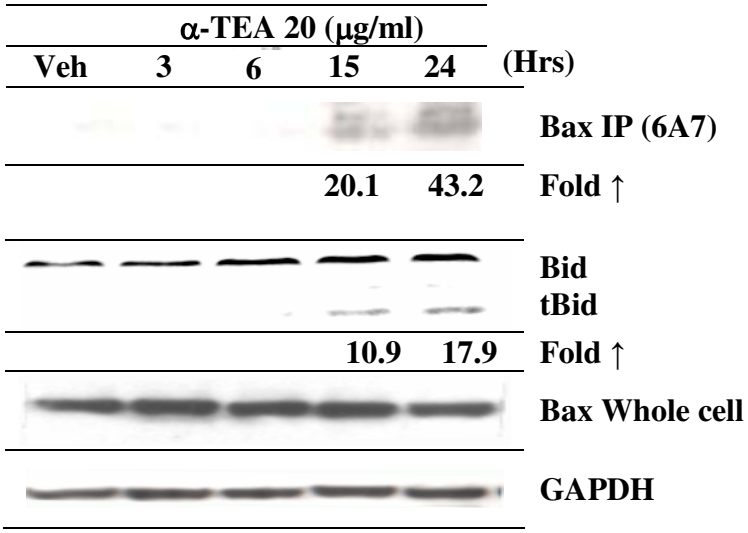


Figure 3.6. α-TEA (20μg/ml) treatment of 66cl-4-GFP cells was able to induce an increase in mitochondrial associated tBid and conformationally active Bax. Results show western immunoblot analysis of tBid , Bad and active Bax over 3,6,15 and 24 hours.

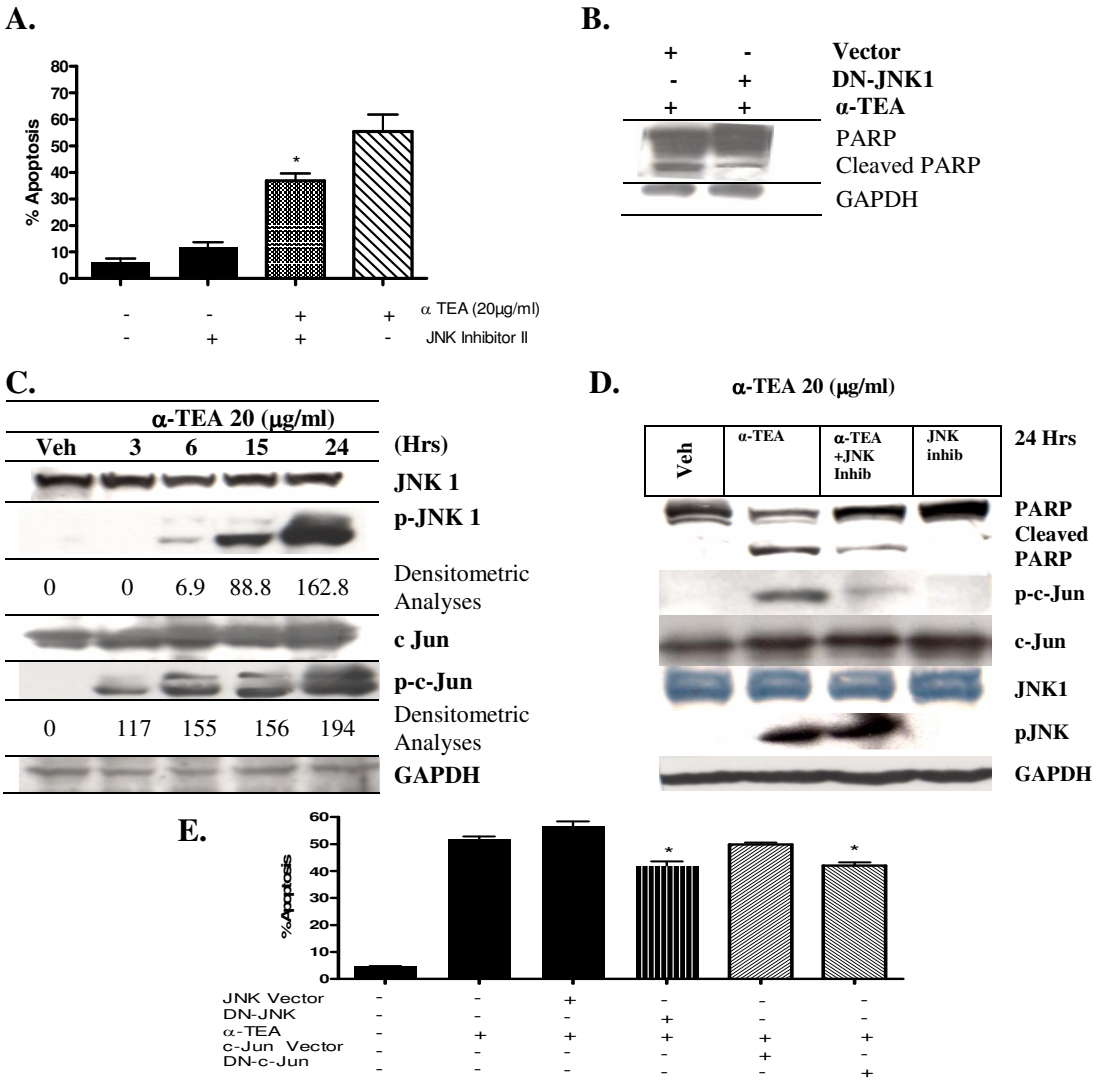


Figure 3.7. JNK plays a pivotal role in in α-TEA induced apoptosis. (A) Cells were treated with 40 nM JNK inhibitor for 2 hours prior to treatment with α-TEA (20μg/ml) for 2 days. Percentage of apoptotic cells was determined via DAPI staining. (B) Western immunoblot to inhibition of JNK 1/2. (C) Western immunoblot to show effect of JNK inhibition on PARP cleavage after treatment with α-TEA (20μg/ml). (D) Western blot shows in increase in phosphorylated JNK and c-Jun following treatment with α-TEA for 3,6,15 or 24 hours. Densitometry values indicate fold increase when compared to vehicle control. (E) Western blot showing effect of JNK inhibitor on downstream target c-Jun and PARP cleavage. (A and B) Data is depicted as the mean ± S.D. of 3 independent experiments. (F) Cells were transiently transfected with double negative JNK or c-Jun and treated with α-TEA (20μg/ml) for 2 days. * designates a significant difference ($P <$ 0.05), when compared to treatment without JNK/c-Jun inhibitor. (D, E and F) are representative of three independent experiments.

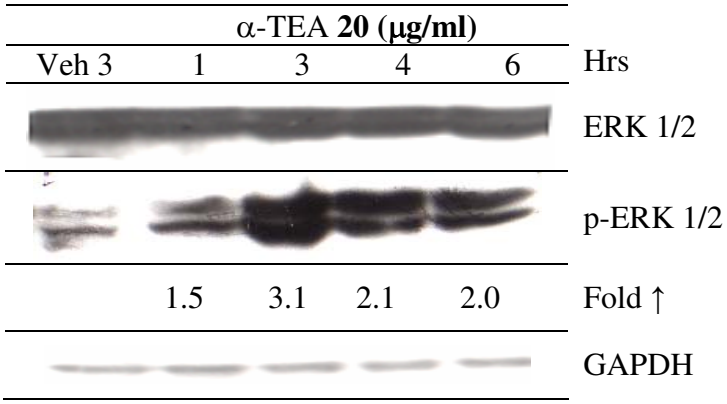


Figure 3.8. Time course activation of ERK1/2 by α-TEA. Cells were treated with α-TEA (20μg/ml) for 1,3,4 and 6 hours (control cells were treated with vehicle for 3 hours) and western immunoblot used to determine protein levels. Data are representative of three independent experiments.

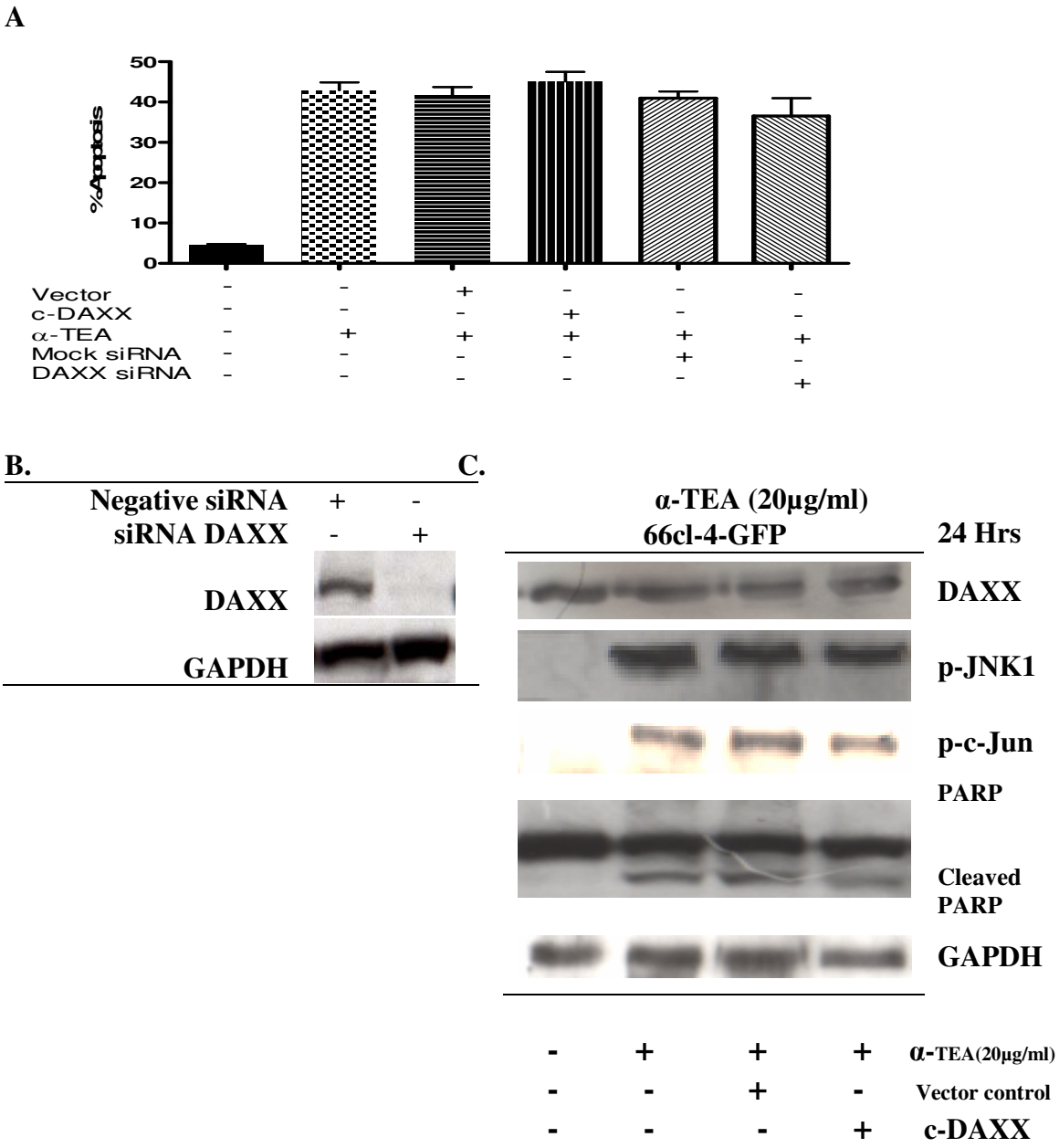
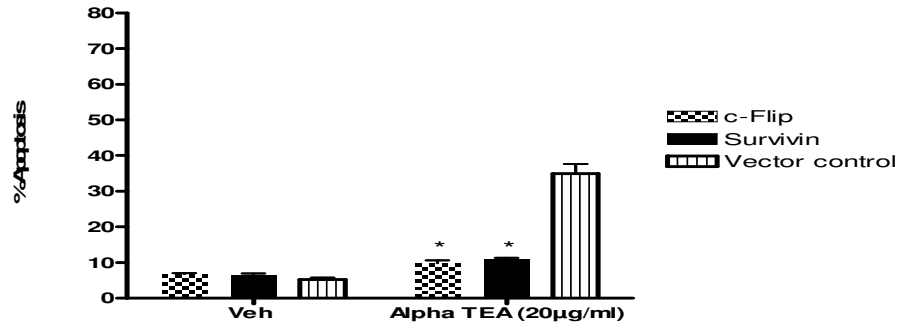


Figure 3.9 Daxx does not appear to play a central role in α-TEA induced apoptosis. (A) Effect on apoptosis by siRNA knockdown of DAXX via siRNA or DN-DAXX was analyzed via DAPI staining. (B) Western immunoblot of knocked down DAXX via siRNA transfection. (C) Cell transiently transfect with DN-DAXX were treated with α-TEA (20µg/ml) for 3,6,15 or 24 hours. Western immunoblot was used to determine effects on associated proteins, JNK,c-Jun and PARP. (A) Data is depicted as the mean ± S.D. of 3 independent experiments. (B and C) Data are representative of three independent experiments

A.



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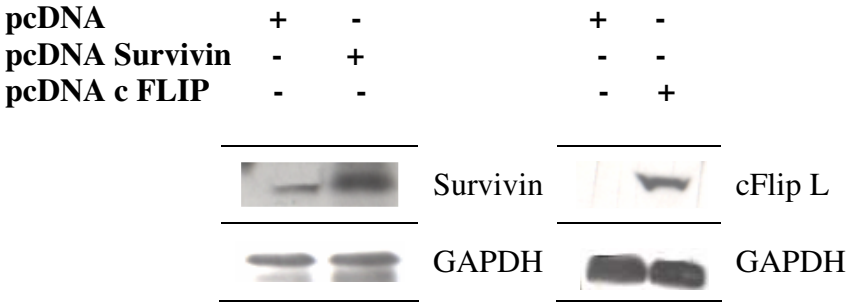

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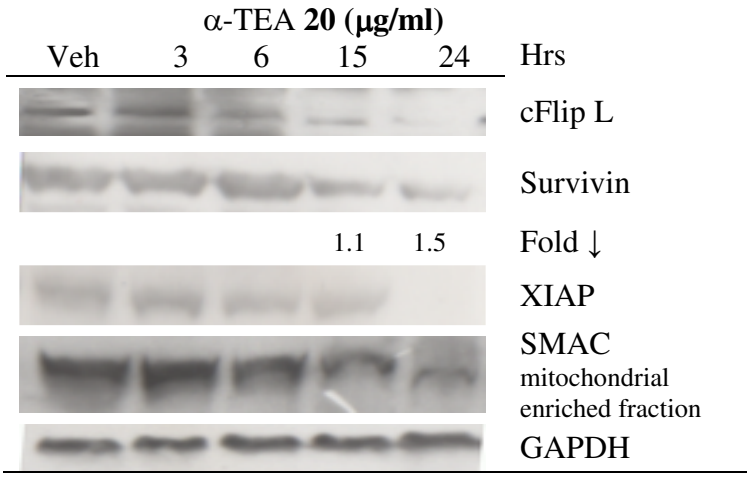
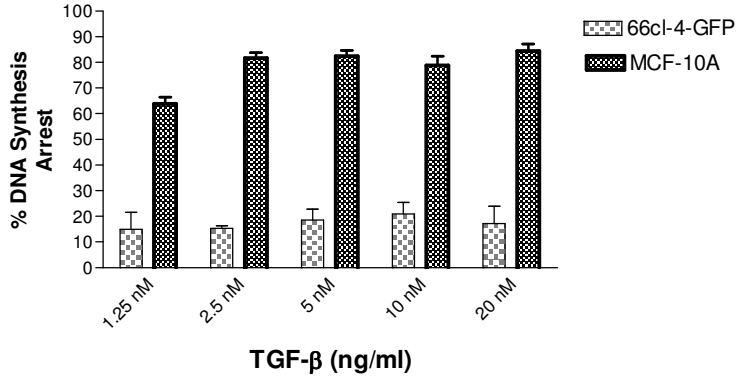


Figure 3.10. c-Flip(L) and Survivin inhibit α-TEA induced apoptosis in 66cl-4-GFP cells. (A) Western immunoblot showing increases in cellular survivin and c-Flip(L) levels following transfection. (B) Effect of c-Flip(L) and survivin overexpression on apoptosis following treatment with α-TEA (20µg/ml) for 2 days. (A) Data are representative of three independent experiments. (C) Effect on c-Flip(L) and following treatment with α-TEA (20µg/ml) for 3,6,15 and 24 hours
Data is depicted as the mean ± S.D. of 3 independent experiments. * = $P < 0.05$ when compared to non-transfected treated control

A.



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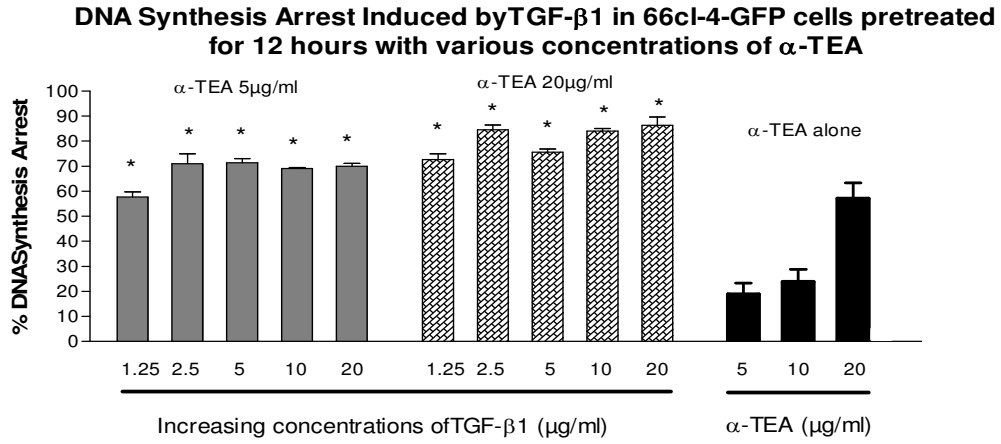


C.

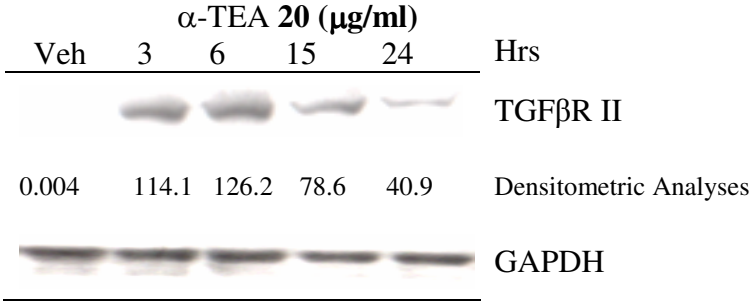
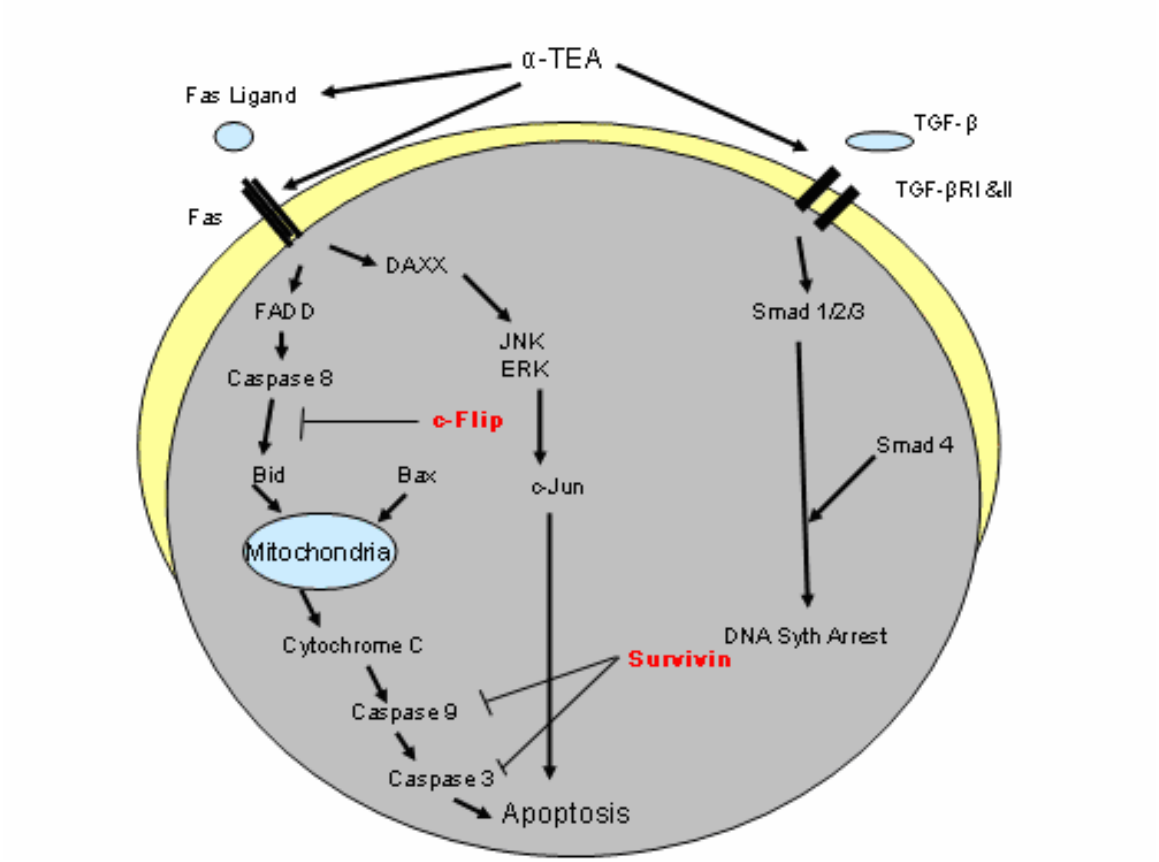


Figure 3.11. (A) 66cl-4-GFP cells are unresponsive to TGF-β1 induced DNA synthesis. (B) Pre-treating 66cl-4-GFP cells with α-TEA for a period of 12 hours shows that sensitivity to TGF-β1 is being restored. (C) α-TEA is shown to increase TGFβR II starting at 3 hours and reaching a maximum level at 6 hours. * = $P < 0.05$ when compared to α-TEA alone



3.12. Role of Inhibitor of apoptosis (IAP) in proposed signaling mechanism of α-TEA induced apoptosis.

Chapter 4: Combination Treatments of α-TEA and Paclitaxel Administered Via Aerosol, Reduced Primary Tumor Burden, Lung and Lymph Node Micrometastasis in a Murine Mammary Model

Abstract

α-TEA has previously been shown to be very effective when used in combination with other chemotherapeutic agents. Combining α-TEA with 9NC (chapter 2) suggested not only that α-TEA can significantly decrease the primary tumor burden when used as part of a combination therapy, but that it may be able to lessen the toxic effects of potentially harmful known anticancer drugs. With this in mind, the studies reported here investigated the effect of using α-TEA following treatment with Taxol on primary tumor burden and metastasis to the lung and lymph nodes. Combination effects on induction of apoptosis within the primary tumor as well as blood vessel density and cellular proliferations were also examined. Combination studies using a daily regiment of α-TEA following two weeks of treatment with Taxol showed significant decreases in not only primary tumor burden when compared to the untreated and liposome control groups (63% and 71% respectively), but also in comparison to treatment groups using α-TEA and Taxol alone. Single treatments of α-TEA and Taxol reduced tumor burden by 38% and 35% when compared to the liposome control group. Combination treatments were significantly better at reducing lung and lymph node micrometastases when compared to control and individual treatment groups (94.8% and 92.3% decrease). Analysis of tumor sections showed combination treatments to significantly decrease tumor blood vessel

density, (P,0.001; mean ± SE 68± 12.5 vs. 219±18.5), cellular proliferation, (P,0.001; mean ±SE 45.1 ± 11.9 vs. 151.1 ± 20.5), and increased the number of TUNEL positive tumor (P,0.001; mean ± SE 103 ± 21.1 vs.11 ± 4.6). Toxicity data show that α-TEA formulated in liposomes and delivered by aerosol (72µg/mouse/day) or gavage (5µg/mouse/day) for 25 days did not cause blood, liver or kidney toxicity. In summary, data supports the use of α-TEA in combination with Taxol as an effective means limiting murine mammary tumor burden and metastases.

Introduction

With cancer being the most common cause of death for women under the age of 85 in the US, the need for continued research and development of effective chemotherapeutic drugs is more important than ever. This year alone there will be an estimated 178,480 women who will be diagnosed with invasive breast cancer and 40,460 breast cancer related deaths. With the evolving nature of breast cancer in mind, the need for a highly effective, non-toxic treatment plan is desperately in need. To meet this need, out lab developed a novel non-hydrolyzable ether linkage attaching an acetic acid moiety to RRR-α-tocopherol, RRR- α -tocopherol, 2, 5, 7, 8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid (abbreviated α -TEA). Previous studies have shown that α-TEA is effective in a wide variety of cancer types including human ovarian, cervivcal, prostate, murine mammary, human breast, lung, lymphoid and colon cancers (25,26,79,108). It has also been shown that α-TEA used *in vivo* is highly effective at reducing primary tumor burden and subsequent metastasis to secondary sites

such as the lungs and lymph nodes. The lungs and lymph nodes are a common site for metastasis and currently pose a significant threat to survival rate in part due to the fact that most therapeutic drugs fail to reach such secondary sites in the required amounts, mainly due to being degraded or inactivated in the liver. In an effort to combat this problem, we are using aerosol treatment in the expectation that the lungs will allow greater absorption of the drug, while at the same time limiting the metabolic degradation that is all too often see in other delivery methods (109-111).

Paclitaxel (Taxol) is an antitumoral drug naturally extracted from the bark of Pacific Yew Taxus Brevifolia. The antitumor activity of paclitaxel has been documented not *only in vitro* but also *in vivo* (112-115). Taxol has a unique mechanism of action when compared to other anticancer drugs in that it causes the polymerization of tubulin. This in turn results in the formation of abnormally stable and nonfunctional microtubules, thereby inhibiting cellular replication in the G0/G1 and G1/M phase. This sequence of events can lead to mitotic arrest, disruption of cell cycle progression and the induction of apoptosis. (57,116-120). Until recently, one main drawback of using Taxol was the problems associated with its low solubility. Previously Taxol was solubilized with the use of polytheoxylated castor oil and ethanol, but this vehicle has been shown to cause hypersensitivity reactions in humans. In response to the need for a more effective delivery method, liposomes were found to be a viable alternative for clinical use of Taxol (121,122)

In the following study we compared the effectiveness of using aerosol Taxol delivered every other day for two weeks, followed by daily aerosol α-TEA treatments, in comparison to untreated and liposome control groups as well as individual α-TEA and Taxol treatment groups. We then looked for significant differences in primary tumor volume, visible lung metastases, lung and lymph micrometastases, tumor cell proliferation, blood vessel density and induction of apoptosis within the primary tumor.

Methods and Materials

α-TEA and Taxol *in vivo* treatments.

α-TEA (FW= 488.8) was prepared for bulk production with high purity (99% pure) and tested for anticancer properties as previously described (26). Taxol (FW= 853.9) was purchased from Sigma Chemical Co., St. Louis, MO.

Animals

Female BALB/cJ mice at 6 weeks of age (20-25 gm body weight) were purchased from Jackson Labs (Bar Harbor, ME). The mice were housed five per cage at the Animal Resource Center at the University of Texas at Austin at 74 ± 2˚F with 30-70% humidity and a 12 h alternating light-dark cycle. Animals were given water and standard lab chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison WI) ad libitum. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Tumor Cell Inoculation

66cl-4-GFP cells were harvested by trypsinization, collected by centrifugation, and re-suspended at a density of 4 x 10^5 cells/100 µl in McCoy's media, that contained no supplements. Mice were injected with 4 x 10^5 cells/ml/100µl in the inguinal area at a point equal distance between the 4^{th} and 5^{th} nipples on the right side using a 23 gauge needle. 60 mice (10 mice/group) were assigned to untreated, liposome, liposome + α-TEA, Taxol + α-TEA, liposome + Taxol and finally Taxol treatment groups. Taxol treatments began 7 days following tumor cell inoculation, and continued every other day for a total of 6 treatments. α-TEA treatments began following termination of Taxol treatments and continued daily until the end of the study (depicted in Fig 4.1). Tumors were measured using calipers every other day, and volumes were calculated using the formula: volume (mm^3) = [width $(mm)^2$ X length (mm)] /2 (81). Body weights were determined on a weekly basis to evaluate possibility of drug toxicity.

Preparation of α-TEA and Taxol Liposomes for Delivery by Aerosol

An α -TEA/liposome ratio of 1:3 (w/w) was prepared as described previously (26). Briefly, the lipid [1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC); Avanti Polar-Lipids, Inc., Alabaster, AL] at a concentration of 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX), then sonicated to obtain a clear solution. α -TEA at 20 mg/ml was dissolved in tertiary-butanol and vortexed until all solids were dissolved. The two solutions were combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α -TEA/liposome. Briefly, stock solutions of Taxol and DLPC were

prepared in tertiary-butanol. Aliquots of Taxol and DLPC (1:10, w/w) were then mixed All treatments were frozen at -80^{o}C and lyophilized overnight to dryness. The formulations were stored at -20^{o}C before use and were reconstituted with sterile water and vortexed until mixed. The initial concentration of Taxol in suspension before nebulization was 1 mg/ml.

Aerosol Delivery

Aerosol was administered to mice as previously described (26, 79). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 L/min airflow was used with an AeroTech II nebulizer (CIS-US, Inc. Bedford, MA) to generate aerosol. The particle size of α -TEA liposome aerosol discharged from the AeroTech II nebulizer was determined by the Anderson Cascade Impactor to be 2.01 µm mass median aerodynamic diameter (NMAD), with a geometric standard deviation of 2.04. About 30% of such particles when inhaled will deposit in the respiratory tract of the mouse and the remaining 70% will be exhaled (79). Prior to nebulization, vials of α -TEA/lipid powder (75 mg/vial) and Taxol/lipid powder (1mg/vial) were brought to room temperature, then reconstituted by adding 3.75 ml and 4ml distilled water respectively, to achieve the final desired concentration of 20 mg/ml α -TEA and 200 µg/ml Taxol. The mixtures were allowed to swell at room temperature for 30 min with periodic inversion and vortexing, and then added to the nebulizer. Mice were placed in plastic cages (7 x 11 x 5 in.) with a sealed top in a safety hood. Aerosol entered the cage via a 1 inch accordion tube at one end and discharged at the opposite

end, using a one-way pressure release valve. The total amount of α –TEA used was therefore 75mg/cage/day and Taxol was 1mg/cage/day. Animals were exposed to aerosol until all of the treatment was aerosolized (approximately 15 min). Approximately 36µg of α-TEA and 23.1µg/g of tissue of Taxol (124) was deposited in the lungs of each mouse per day.

Lung and Lymph Node Metastasis.

Visible macroscopic metastases were counted in all five lung lobes at time of sacrifice. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; 200 X magnification) as previously described (26). For micrometastatic analyses, the left lung lobe was flattened and the top and bottom surfaces were scored for fluorescent microscopic metastases. Fluorescent microscopic metastases were scored by size into three size grouping: < 20 µm, 20-50 µm, and >50 µm. Based on a typical 66cl-4-GFP tumor cell size of 10-20 µm in diameter, the < 20 µm grouping is thought to represent solitary cells; the 20-50 µm grouping two to five cells; and the > 50 µm grouping microscopic metastases of greater than two to five cells

Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling Assay for Detection of Apoptosis *in Vivo*.

Deparaffinized sections (5 µm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag In Situ Apoptosis Detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Nuclei that stained brown were scored

as positive for apoptosis and those that stained blue were scored as negative. At least sixteen 400X microscopic fields were scored per tumor. Digital images were captured using an Olympus DP71 digital camera and DP controller software. Data are presented as the mean ± S. E. number of apoptotic cells counted in ten separate tumors from each group.

Ki-67 immunohistochemical staining for detection of proliferation *in Vivo*.

Deparaffinized sections (5 µm) of tumor tissue were used to assess proliferation, using antibody to the Ki-67 antigen, which is a nuclear antigen expressed in proliferating cells and which serves as an indicator of the number of cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 minutes followed by washing with PBS. Rabbit serum (10 %) in PBS was applied to 5 micron tumor tissue sections in order to block non-specific antibody binding, prior to incubating with primary antibody (rat-anti-mouse Ki-67 antibody; 1:200 dilution, DAKO Corp., Carpinteria, CA) overnight at 4°C. After primary antibody incubation, washed slides were incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 minutes at room temperature. Sections were then incubated with avidin-biotin complex (ABC-HRP, Vector Laboratories) for 30 minutes at room temperature. Immunoreactivity was visualized via incubation with di-aminobenzidine dihydrochloride (DAB). Slides were lightly counterstained with hematoxylin. Ki-67 positive stained (brown) cells were counted in five separate fields

per sample. Digital images were captured using an Olympus DP71 digital camera and DP controller software

CD31 staining for determination of blood vessel formation *in vivo*.

Immunohistochemistry was used to assess the presence of the endothelial antigen CD31 as an indicator of small capillaries in primary tumor tissue. Deparaffinized tissue sections were pre-treated with 0.06% Protease Type XXIV (Sigma) for 10 minutes at room temperature prior to incubation with CD31 (PECAM) antibody (PharMingen, San Diego, CA) at a 1:400 dilution overnight at 4 °C. Detection utilized the Tyramide Signal Amplification Biotin System - Peroxidase (PerkinElmer Life Sciences, Boston, MA) with di-aminobenzidine dihydrochloride development. For contrast, the sections were lightly counterstained with hematoxylin. Entire tumor sections were scored for CD31 stained vessels (brown), and adjusted for tumor size, by dividing number of CD31 vessels by length X width of tumor tissue mounted on slide. Digital images were captured using an Olympus DP71 digital camera and DP controller software.

Toxicity Analysis

6 week old Balb/cJ female mice were treated with α-TEA formulated in liposomes for 25 days. The following groups (5 mice/group) were evaluated. Untreated control, liposome only by gavage, liposome only by aerosol, α-TEA (5mg/day) administered by gavage and α-TEA (72μg/day) by aerosol. At the completion of treatments, blood cells and serum

were tested for bone marrow, liver and kidney toxicity. Note, toxicity analysis were conducted by the U.T. M.D. Anderson Cancer Center at Smithville, Texas.

Statistical Analyses

Animal numbers for experiments were determined by power calculations derived from data generated by preliminary pilot studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance using SPSS (SPSS, Inc, Chicago, IL). Difference in number of fluorescent microscopic metastases per group was determined using the Mann-Whitney rank test using Prism software version 4.0 (Graphpad, San Diego, CA). A level of $P< 0.05$ was regarded as statistically significant.

Results

α-TEA used following treatment with Taxol significantly reduced primary tumor burden when compared to individual treatment groups

The mean tumor volume of the Taxol, Liposome + α-TEA and Taxol + α-TEA treatment groups were all significantly reduced when compared to the liposome control or untreated group ($P<0.001$; means ±SE 394±115 mm^3; 380±99; 178±49 mm^3 and 609±176 mm^3 respectively) during 21 days of treatment (Fig. 4.2). The Taxol + α-TEA combination treatment group showed a mean tumor volume that was significantly lower than

individual α-TEA or Taxol groups (P<0.001; mean ±SE 178±49 mm^3, P<0.001: 380±99, P<0.001: 394±115, P<0.001: 310±102 respectively)

Animal Weights

Over the duration of the study the animal weight for each treatment group showed no significant overall difference using the Mann-Whitney rank test (Fig 4.3). The use of aerosol Taxol showed no obvious toxicity when compared to previous studies using IP injection (not shown).

Combinations of α-TEA + Taxol Reduced Visible Lung Metastasis.

Upon completion of the study, animals were euthanized and all five lung lobes from each mouse were examined. 10 out of 10 and 9 out of 10 mice in the untreated and liposome control groups scored positive for visible macroscopic metastasis. α-TEA (6/10), Taxol + Liposome (4/9), Taxol only (3/10) and Taxol + α-TEA (2/10). Total number of visible lung metastases were significantly reduced in Taxol and α-TEA groups when treated separately and in combination (Table 4.1).

Individual treatments and α-TEA/Taxol combination treatments significantly reduced lung and lymph node micrometastatic lesions

Green fluorescing lung and lymph micrometastatic lesions in control and treatment groups were grouped into three size categories: <20µm, 20-50µm and >50µm. The data showed a significant decrease in total number of microscopic lung metastases in all

treatment groups (α-TEA alone, Taxol + α-TEA, Taxol + liposome, Taxol alone) in comparison to the two control groups (untreated and liposome) (P<0.0001; 41.5±3.7, 6.5±1, 29.6±4.6, 27.7±3.1 vs. 125.9±16.6, and 1225. ±19.7) (Fig 4.4A). There were 94.8% fewer total micrometastatic lung lesions in the Taxol + αTEA group compared to controls. The liposome +α-TEA, Taxol +liposome and Taxol treatment groups had 66.9%. 76.4% and 78.6% fewer micrometastatic lesions than controls respectively. All three size categories and total number of micrometastatic lesions in the Taxol +α-TEA combination group were significantly different from individual treatment groups (P<0.0001) (Fig 4.4A).

Green fluorescent microscopic metastases in the axillary and brachial lymph nodes from each treatment group were counted. All treatment groups (α-TEA alone, Taxol + α-TEA, Taxol + liposome, Taxol alone) exhibited a significant reduction in total metastases when compared to the control groups (untreated and liposome), (P<0.0001; 95.4±16.7, 11.3.4±3.0, 69.7±8.1, 77±12.5 vs. 147.4±15.4 and 139±21.2) with Taxol + α-TEA having 92.3% fewer total micrometastatic lymph lesions in comparison to the control group (P<0.0001; 11.3±3.0 vs. 147.4±15.4), (Fig. 4.4B). All three size categories and total number of lymph node micrometastatic lesions in the Taxol + α-TEA treatment group were significantly reduced when compared to the α-TEA or Taxol individual treatment groups ($P<0.0001$). The individual treatment groups showed 35.3%(liposome + α-TEA), 52.7%(Taxol + Liposome) and 47.5%(Taxol) fewer micrometastatic lymph lesions in comparison to control (P<0.0001; 95.4±16.7, 52.7.4±8.3, 47.5±12.5).

α-TEA and Taxol Treatments Induced Tumor Cells to Undergo Apoptosis.

TUNEL staining of 5 µm tumor sections were used to evaluate the induction of apoptosis by the treatment groups *in vivo*. The mean ± SE number of TUNEL positive cells/field for liposome + α-TEA, Taxol + α-TEA, Taxol + Liposome and Taxol treatment groups were significantly increased 30.1 ± 11.6, 103 ± 21.1 and 61 ± 10.2 and 59 ± 10.9 when compared to the aerosol control groups which were 11 ± 4.6 and 13± 3.1 TUNEL positive cells per field (*P<0.0001*). The combination group showed significantly more apoptotic cells per tumor than any of the individual groups (Fig. 4.5A).

α-TEA and Taxol alone and in combination significantly reduced blood vessel density.

The ability of all four treatment groups to effect blood vessel density was determined via staining for the CD31 (PECAM-1) endothelial cell marker. All treatment groups (Liposome + α-TEA, Taxol + α-TEA, Taxol + Liposome, Taxol) significantly reduced the total number of CD31 positive stained cells (P<0.0001, 129± 12.6 , 68± 12.5, 142± 13.1, 140±22.8 vs. 219±18.5). The cotreatment group was significantly different in all three size categories (*P<0.0001*), as well as an total reduction when compared to the α-TEA or Taxol treatment groups (P<0.0001, 68± 12.5 vs 129± 12.6 and 142± 13.1). It should be noted that liposome + α-TEA and Taxol + α-TEA treatment groups were effective at reducing overall blood vessel size (Fig. 4.5B).

Combination of Taxol and α-TEA significantly reduced cellular proliferation when compared to control and individual treatment groups alone.

The ability of the treatments to inhibit cell proliferation were determined by examination of tumor immunohistochemically stained for the nuclear antigen Ki-67, a biomarker for proliferation. The mean ± SE number of positive cells per field for tumors taken from each group were: liposome + α-TEA ($P<0.001$; 122.8 ± 26.5); Taxol + α-TEA ($P<0.001$; 45.1 ± 11.9), Taxol + Liposome ($P<0.0001$; 66.5 ± 12.6) and Taxol ($P<0.001$; 76.2 ± 18.5) compared to tumors from the aerosol control groups (means ± SE of 151.1 ± 20.5 and 121±14.2). The Ki-67 positive cells for tumor sections from mice treated with α-TEA alone did not show a significant difference from liposome treatment group, but when used in combination with Taxol Ki67 positive cells were significantly different from the control groups as well as individual treatment groups (Fig. 4.5C).

Discussion

This study suggest that using α-TEA following treatment with Taxol is an effective, non-toxic agent in inhibiting primary tumor burden, visible metastasis, lung and lymph node micrometastases, cellular proliferation, and blood vessel density in a syngeneic mouse model. It should also be noted that the combination treatment was also significantly effective in inducing tumor cells to undergo apoptotic cell death as determined via TUNEL staining.

Previous studies have indicated the limiting factor when using Taxol is its toxicity, and as a direct result the need for a non-toxic dosage and delivery method is paramount in the continued investigation of this drug's benefits. This study was able to use an effective drug delivery system that showed to obvious signs of toxicity to the animals. The results shown here mirror data from previous studies that suggest using α-TEA in conjunction with known anti-cancer drugs is a highly effective treatment plan. α-TEA has been show to be effective when used with known chemotherapeutic agents such as celecoxib and 9-nitrocamptothecin against breast cancer and cisplatin against ovarian cancer (79,81, 82). This highlights the need for continued investigation of α-TEA. The data was also consistent with earlier studies performed in our lab, which effectively show that α-TEA alone significantly reduces tumor burden in similar mouse models (26, 79, 82).

In conclusion, the data presented in this study shows that α-TEA when used in combination with other anticancer drugs, is an effective in reducing tumor burden and metastases. It is believed that the effectiveness of this treatment may further be improved with repeated doses of Taxol followed by treatment of α-TEA. Taken together, these results hold a great deal of promise in the use of α-TEA as an effective treatment against aggressive forms of mammary cancers

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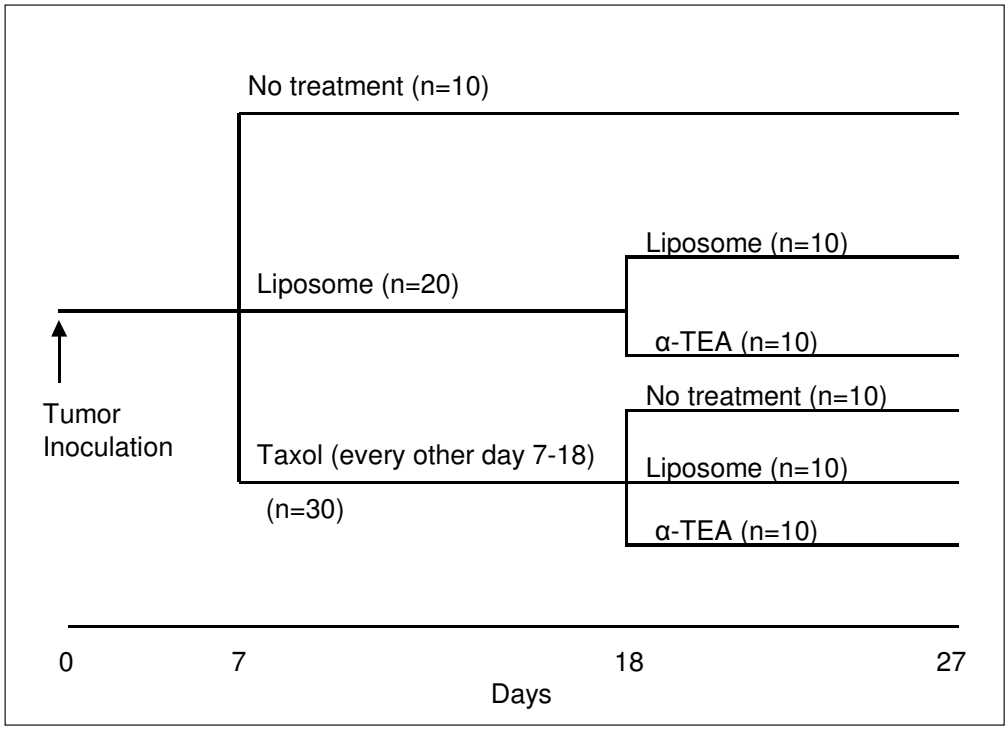


Figure 4.1: Treatment groups and schedule for duration of study. Briefly, animals were separated into treatment groups 7 days after tumor cell inoculation. Untreated and liposome groups served as control groups. 30 mice received Taxol, every other day for a total of 6 treatments and then were split into untreated, α-TEA or liposome groups. α-TEA treatment was continued until termination of study.

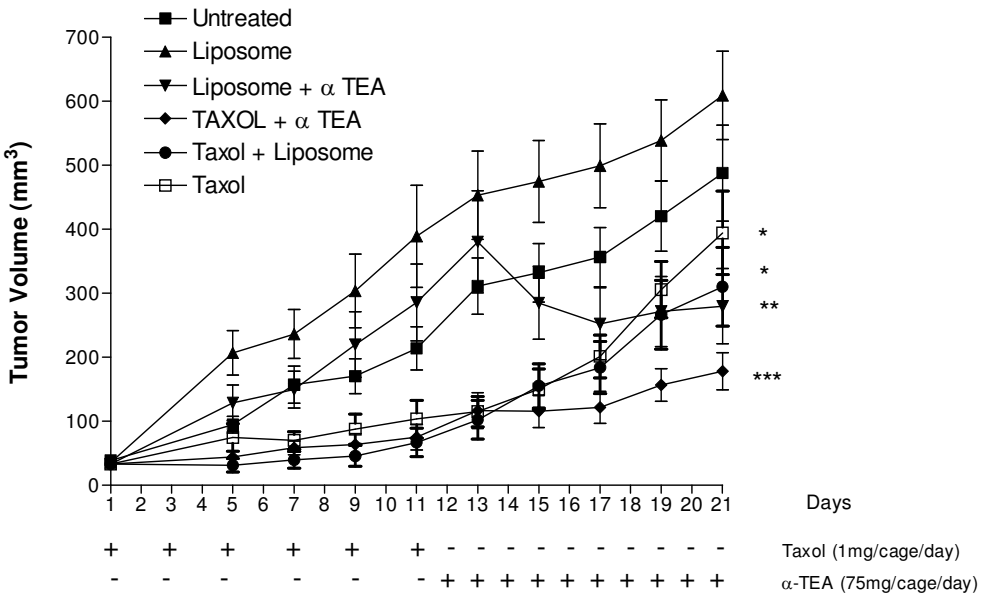


Figure 4.2: Aerosolized α-TEA, Taxol, and combination treatments reduced tumor volume. Taxol treatments started 7 days after subcutaneous injection of $4x10^5$ 66cl-4-GFP cells/mouse and continued every other day for 6 treatments. α-TEA (36µg/mouse/day) treatment started on day 18 and continued daily for 10 days
* significantly different from untreated and liposome group; P<0.05
** significantly different from liposome treatment group; P<0.05
*** significantly different from untreated, liposome and individual treatment groups; P<0.05

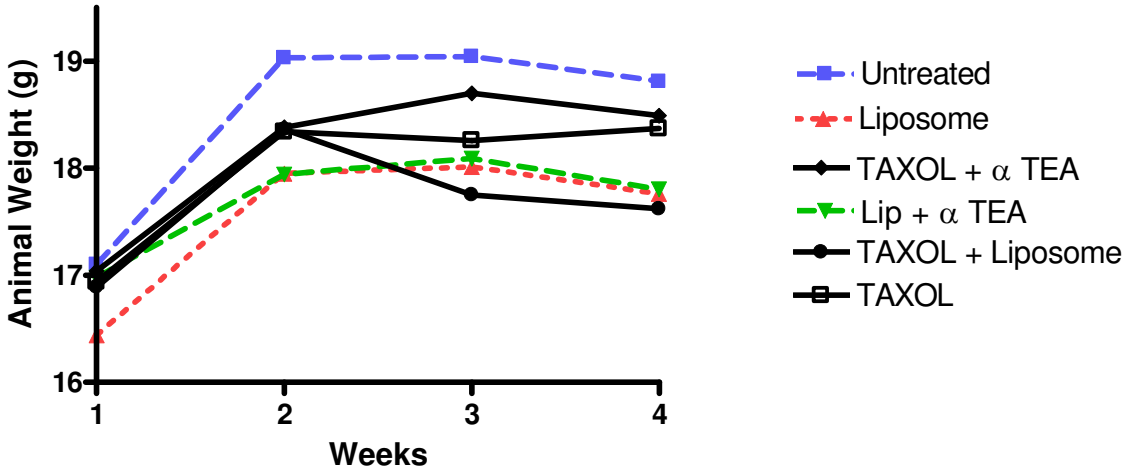


Figure 4.3: Animal weights of the six treatment groups were determined over the duration of the study. Animal weights were measured every other day after the first week of the study

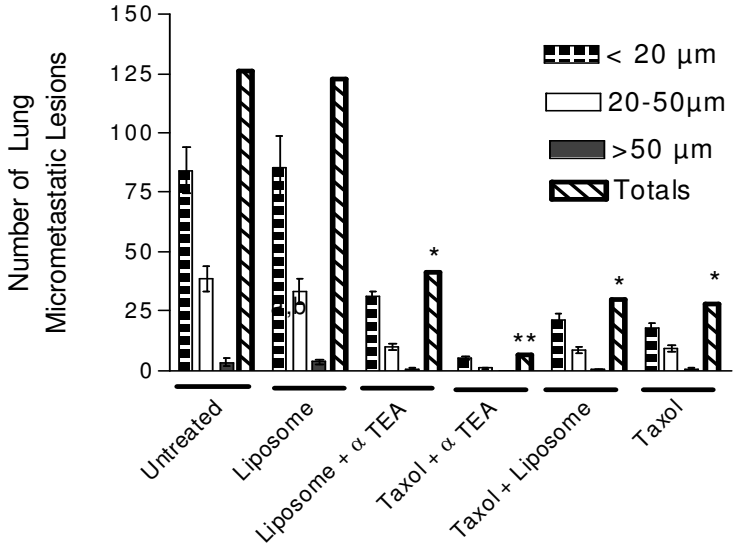
Table 4.1:

Evaluated the number of visible lung metastases in control and treatment groups.

Treatments Delivered via aerosol	# of animals/group showing macroscopic lung metastasis [a]	Total # of macroscopic lung tumor foci [b]
1. Untreated	10/10	24
2. Liposome	9/10	26
3. Liposome + α TEA	6/10	7*
4. Taxol + α-TEA	2/10 *	2*
5. Taxol + Liposome	4/9 *	5*
6. Taxol	3/10 *	4*

[a] Number of macroscopic metastatic lesions in all five lung lobes in each animal in control and treatment groups were counted at the time of euthanasia. Data is depicted as number of mice/group with visible lung metastasis.
[b] Data represents the total number of visible lung macroscopic metastasis observed in each group.
* Designates significant difference when compared to untreated or liposome control ($P<0.05$)

A.



B.

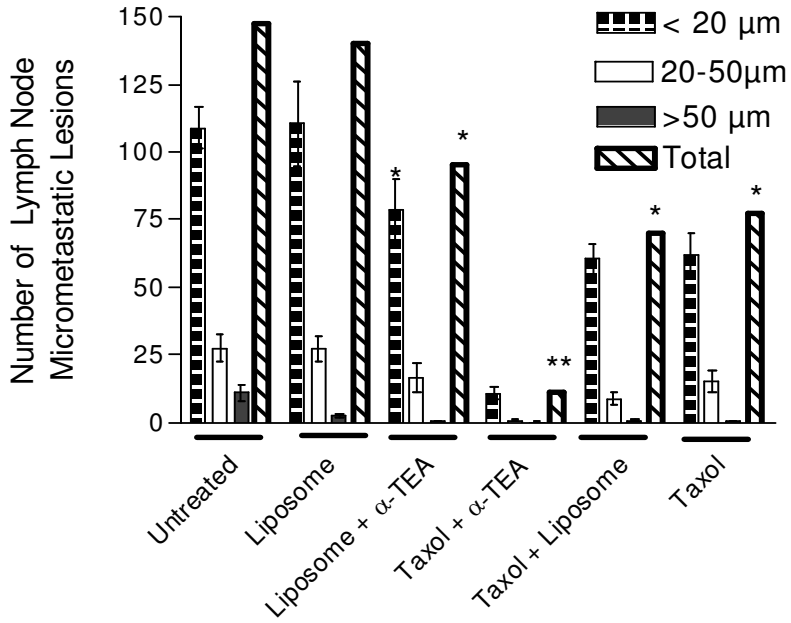


Figure 4.4: α –TEA, Taxol and combinations aerosol treatments exhibited a significant decrease in (A) lung and (B) lymph node metastasis.
* significantly different from control group; P<0.05
** significantly different from control and individual treatment groups; P<0.05

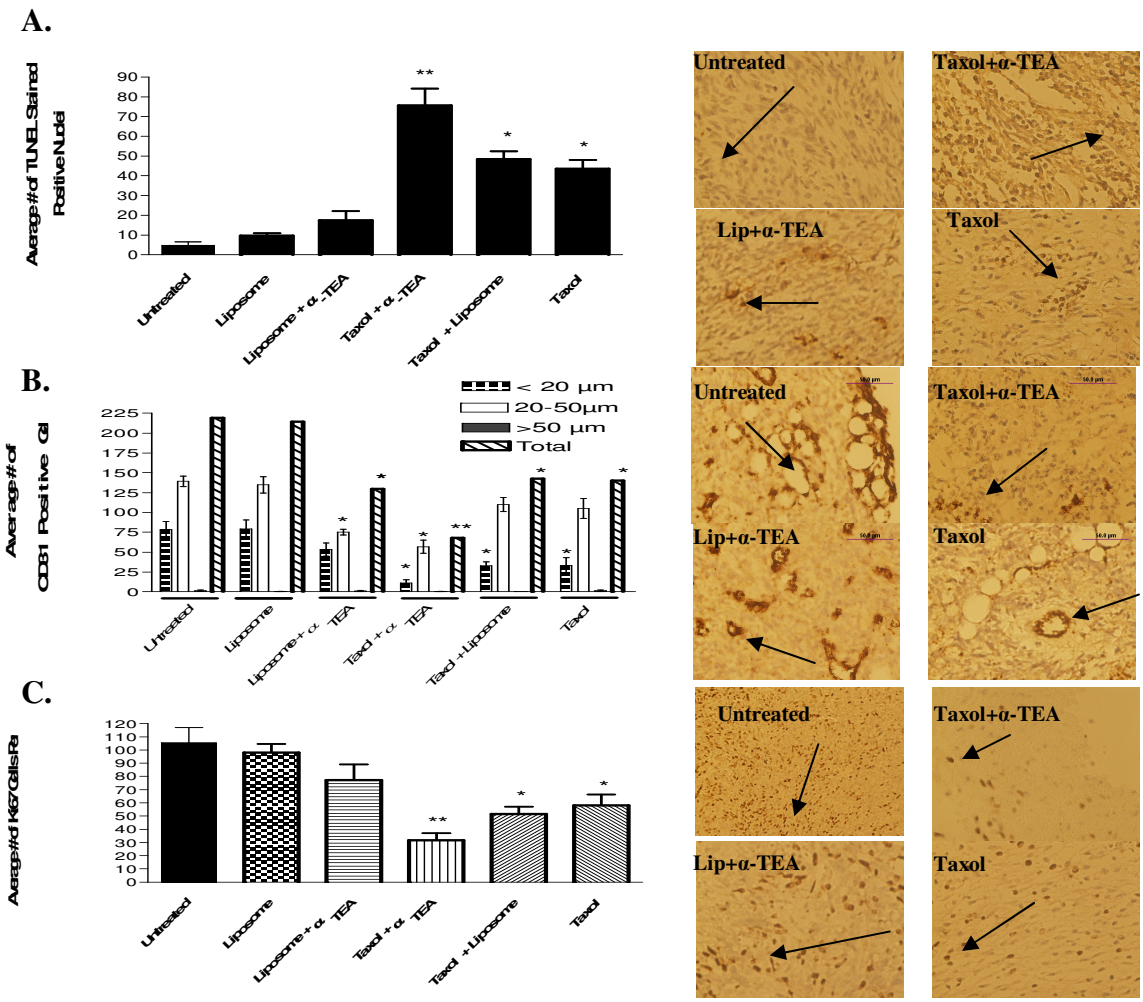


Figure 4.5: (A) α-TEA and Taxol induction of apoptosis in vivo was investigated using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. TUNEL-positive nuclei were counted at 400X magnification. All positive nuclei were counted in 16 separate fields on each slide. Ten separate tumors from each group were counted. (B) α-TEA, Taxol + α-TEA and Taxol treatment groups showed an inhibitory effect on angiogenesis as determined by CD31 staining, when compared to control ($P<0.0001$, 129± 12.6 , 68± 12.5, 142± 13.1, 140±22.8 vs. 219±18.5). Blood vessels were identified using the CD31 antibody where 5µm tumor sections were counted for CD31-positive staining. (C) Both combination and individual treatments of α-TEA and Taxol, induce inhibition of 66cl-4-GFP cell proliferation *in vivo*. Cell proliferation was determined using 5 µm tumor sections derived from tumors from all six treatment groups. Proliferating cells were identified using Ki-67 antibody. 5 separate fields of each slide were scored for Ki-67 positive cells. Data are mean ± S.E. of all tumors in each group. Cells were counted with 400X magnification using a Zeiss ICM 405 fluorescent microscope under visual light.

* significantly different from control group; $P<0.05$

** significantly different from control and individual treatment groups; $P<0.05$

Chapter 5: Summary and Future Directions

Summary

Breast cancer continues to be the most common type of cancer in women and the second most common cause of cancer death in women in the U.S. Over the past few decades a better understanding and awareness of the risks associated with breast cancers has led to a continual increase in the number of women who undergo mammography for screening, in turn leading to more cancers being caught at an early stage and thus improving the long terms survival rates. Even with this increase in preventative measures, breast cancer is still the most common cause of death in women between the ages of 45 and 55. Over the past 40 years research has yielded a vast array of information about the causes of breast cancers. Epigenetic as well as genetic factors are known to play crucial roles in the initiation, promotion and eventual progression of many different types of breast cancer. Today staging systems have been developed to allow doctors to characterize the extent to which a particular cancer has spread and to allow them to make more informed decisions concerning treatment options. Such treatment options for breast cancer may involve surgery (removal of the cancer alone or, in some cases, mastectomy), radiation therapy, hormonal therapy, and/or chemotherapy.

With continued advances in screening, diagnosis, and treatment, the death rate for breast cancer has declined by about 20% over the past decade, and research continues in the hope that more effective screening and treatment programs will be developed. As more is learned about the heterogeneic nature of all types of cancer, it becomes increasingly

evident that in order to tackle the seemingly limitless defects in tumor cellular regulation, there is a desperate need for effective, non-toxic combination treatments, in the hope to reduce cancer cell survival via multiple approach methodology. The goal of these studies was to determine the effectiveness of α-TEA alone and in combination with 9NC and Taxol to induce cell death in an aggressive murine mammary cancer cell line.

Until the synthesis of α-TEA, RRR-α-tocopherol succinate (VES, vitamin E succinate) was considered to be the most effective form of vitamin E capable of inhibiting tumor cell growth. Studies suggested that the tumor inhibitory functions of VES were due to the intact form of VES being maintained, as cleavage of the succinic acid moiety from RRR-α-tocopherol succinate, led to a loss in its ability to exert its anti-cancer abilities . As a direct result of these findings a novel form containing a non-hydrolyzable ether linkage attaching an acetic acid moiety to RRR-α-tocopherol was developed. To date α-TEA has been shown to induce similar effects on cancer cells as was previously shown with VES (25, 140). The studies presented here further demonstrate the unique ability of α-TEA to be used as a compelling chemotherapeutic agent.

Chapter 2 investigated the ability of α-TEA when used in combination with 9NC to reduce primary tumor burden and macro and micrometastatic lesions as well as its effect on tumor cell proliferation, apoptosis and angiogenesis, when compared to individual treatment groups *in vivo*. Combination treatments were more effective at reducing tumor volume, inducing tumor cell apoptosis and decreasing tumor cell proliferation when compared to control treatments. In vitro studies helped us gain a better understanding of

how exactly α-TEA along and in combination with 9NC is able to induce tumor cell destruction via apoptosis. The results showed that combination treatments were able to stimulate a Fas, JNK and mitochondrial dependent apoptotic signaling pathway. In a time dependent fashion, α-TEA and 9NC were able to up-regulate pro-death player while at the same time decrease the effectiveness of pro-survival players such as c-Flip(L) and Survivin. This two pronged approach gives us an insight in the multiple ways in which α-TEA is able to shut down tumor cellular functions.

Chapter 3 Investigated the mechanism of action of α-TEA in its mediation of tumor cell death via apoptosis in 66cl-4-GFP murine mammary cells *in vitro.* It was discovered the α-TEA's induction of apoptosis is Fas dependent. Treatment of α-TEA was shown to increase membrane levels of Fas as well as increase Fas ligand. Initiation of cellular death was also dependent on caspases 8, 9 and 3 as was seen when their function was inhibited. Together this data suggests a type II, mitochondrial dependent induction of apoptosis. Further investigation revealed that JNK and c-Jun are playing critical roles in the ability of α-TEA to cause cell death. ERK was also activated (pERK1/2) and it is crucial to whether ERK stimulates cell death or cellular survival. Previous work in our lab in other cell lines has shown the importance of DAXX to α-TEA induced apoptosis. These studies suggest that DAXX plays no critical role in the ability of α-TEA to initiate cell death in this cell line. Finally α-TEA was able to restore sensitivity to TGF-β in 66cl-4-GFP cells. The ability to up-regulate TGF- βRII helps us gain a better understanding of the multiple ways in which α-TEA can determine cell fate. The ability of α-TEA to not only affect pro-death players, but also pro-survival proteins is an exciting prospect as it

helps us understand how α-TEA has the ability to affect cellular fate from two different approaches.

Chapter 4 focused on the effectiveness of an aerosol Taxol + α-TEA treatment in a syngeneic mouse model in comparison to individual treatments alone. The results showed that when Balb/cJ mice were first treated with aerosol Taxol for two weeks (treated every other day), and then treated with α-TEA in aerosol form daily, there were a significant differences in tumor volume, macro and micrometastatic lung lesions as well as micrometastatic lymph node lesions, when compared to the individual treatment groups. The combination treatment was significantly better at inducing tumor cell apoptosis as was seen via TUNEL staining, decreasing tumor cell proliferation (Ki67) and decreasing blood vessel formation and density (CD31). Taken together, these data suggest that using α-TEA in conjunction with Taxol administered via aerosol is a very effective and non-toxic treatment program.

Future Directions.

The data presented here suggest that using α-TEA in conjunction with known chemotherapeutic drugs is not only highly effective, but may also aid in reducing the potential for toxicity that many anticancer drugs such as 9NC and Taxol possess. The in vitro studies described here help to shed light on potential apoptotic signaling pathways used by α-TEA to cause tumor cell death. With this information in hand, future studies should include more *in vitro* and *in vivo* work in this field. The *in vitro* approach will focus on cellular mechanism by which α-TEA and Taxol are able to induce a more

effective anti-tumor response than either treatment alone. Based on the current literature, one theory is that Taxol is working via similar apoptotic pathways to α-TEA in that is can modulate Fas/Fas ligand expression, while at the same time regulate mitogen-activated protein kinases, and downregulate the pro-survival pathway mediated via Akt/Survivin/XIAP (Fig. 5.1). Clearly more work needs to be done to understand the mechanistic intricacies of combination therapy. Future *in vivo* work will examine the ability of multiple doses of Taxol and α-TEA to prevent tumor cell volume from bouncing back as time progresses. These studies will be aided by determining the maximum tolerable does of aerosol Taxol that can be given, while still remaining non-toxic to the treated animals.

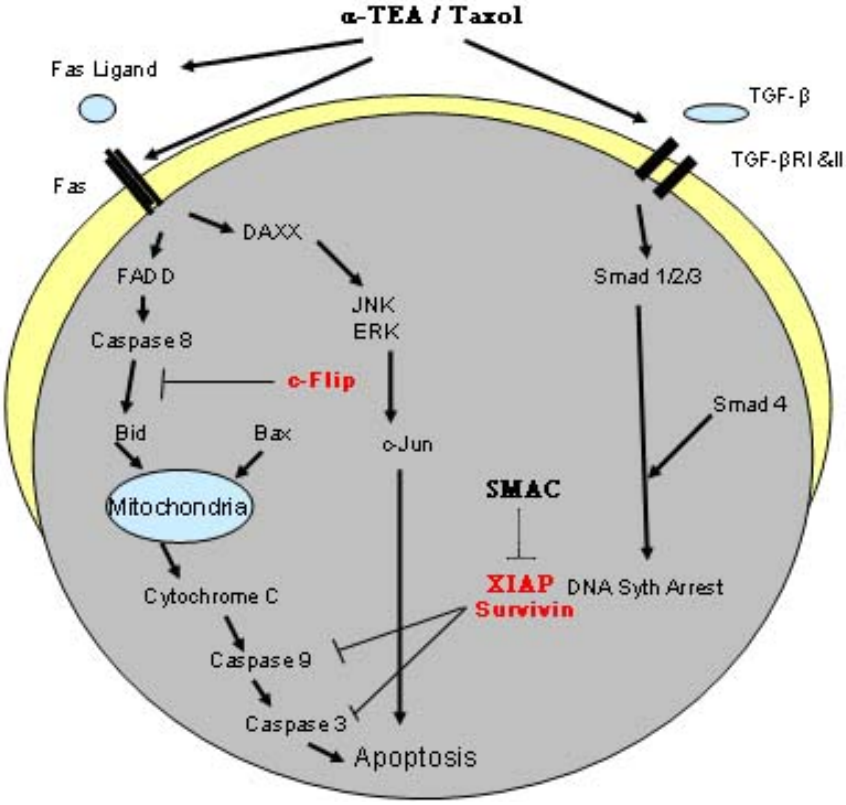


Figure 5.1. Proposed apoptotic signaling mechanism following treatment with α-TEA + Taxol

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Vita

Paul Brian Latimer was born in Kingston-upon-Thames, Surrey England on January 13, 1976 to Brian and Ellen Latimer. He has one older sister Julie who was born on December 30, 1973. Paul's family moved to Edinburgh just after his first birthday in 1977, where he lived until graduating from George Heriots School, Edinburgh, in 1993. In the summer of that year he moved to Canberra Australia, to train at the national institute of sport. In 1994 he was awarded an athletic scholarship to the University of Texas at Austin, were he moved that fall to begin his secondary education. He received his Bachelor of Science in Molecular Biology in 2000 and the following year a Bachelor of Science in Zoology both from the University of Texas. In the spring of 2002 he began his graduate career in the lab of Drs. Bob G Sanders and Kimberly Kline were he remained throughout his graduate career.

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