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Targeting breast cancer with natural forms of vitamin E and simvastatin

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Targeting breast cancer with natural forms of vitamin E and simvastatin

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Dedicated to my parents...

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Targeting breast cancer with natural forms of vitamin E and simvastatin

Archana Gopalan, Ph.D.
The University of Texas at Austin, 2012

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Breast cancer is the second leading cause of death due to cancer in women. A number of effective therapeutic strategies have been implemented in clinics to cope with the disease yet recurrent disease and toxicity reduce their effectiveness. Hence, there is a need to identify and develop more effective therapies with reduced toxic side effects to improve overall survival rates. This dissertation investigates the mechanisms of action of two natural forms of vitamin E and a cholesterol lowering drug, simvastatin, as a therapeutic strategy in human breast cancer cells. Vitamin E in nature consists of eight distinct forms which are fat soluble small lipids. Until recently, vitamin E was known as a potent antioxidant but emerging work suggests they may be resourceful agents in managing a number of chronic diseases including cancer. Anticancer properties of vitamin E have been identified to be limited to the γ- and δ- forms of both tocopherols and tocotrienols. Gamma-tocopherol (γT) and gamma-tocotrienol (γT3) have both already been identified to induce death receptor 5 (DR5) mediated apoptosis in breast cancer cells. Studies here show that similar to γT3, γT induced DR5 activation is mediated by c-Jun N-terminal

kinase/C/EBP homologous protein (JNK/CHOP) proapoptotic axis which in part contributed to γT mediated dowregulation of c-FLIP, Bcl-2 and Survivin. Also, both agents activate *de novo* ceramide synthesis pathway which induces JNK/CHOP/DR5 proapoptotic axis and downregulates antiapoptotic factors FLICE inhibitory protein (c-FLIP), B-cell lymphoma 2 (Bcl-2) and Survivin leading to apoptosis. Simvastatin (SVA) has been identified to display pleiotropic effects including anticancer effects but mechanisms responsible for these actions have yet to be fully understood. In this dissertation, it was observed that simvastatin induced apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 proapoptotic axis and down regulation of antiapoptotic factors c-FLIP and Survivin which are in part dependent on JNK/CHOP/DR5 axis. The anticancer effects mediated by simvastatin can be reversed by exogenously added mevalonate and geranylgeranyl pyrophosphate (GGPP), implicating the blockage of mevalonate as a key event. Furthermore, work has been done to understand the factors responsible for drug resistance and identify therapeutic strategies to counteract the same. It was observed that development of drug resistance was associated with an increase in the percentage of tumor initiating cells (TICs) in both tamoxifen and adriamycin resistant cells compared to their parental counterparts which was accompanied by an increase in phosphorylated form of Signal transducer and activator of transcription 3 (Stat3) proteins as well as its downstream mediators c-Myc, cyclin D1, Bcl-xL and Survivin. Inhibition of Stat3 demonstrated that Stat3 and its downstream mediators play an important role in regulation of TICs in drug resistant breast cancer. Moreover, SVA, γT3 and combination of SVA+γT3

has been observed to target TICs in drug resistant human breast cancer cells and downregulate Stat3 as well as its downstream mediators making it an attractive agent to overcome drug resistance. From the data presented here, the mechanisms responsible for the anticancer actions of γT, γT3 and SVA have been better understood, providing the necessary rationale to test these agents by themselves or in combination in pre-clinical models.

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Chapter 1

Introduction

1.1 Breast cancer

Cancer is a leading cause of death among people in the United States. It is characterized by uncontrolled cellular proliferation and metastasis to distant sites. Breast cancer is the second largest cause of death in U.S. women due to cancer. In 2012, 226,870 new cases of breast cancer have been predicted to be identified in women and claim nearly 39,510 lives [1]. Present treatment options available to women in the include surgery, radiation therapy, hormonal therapy, chemotherapy and targeted therapy [2]. Often these options are applied in adjuvant settings to achieve enhanced effects. Yet, these treatment strategies are limited by their side effects and toxicity [2, 3]. Recently, statins, nutrients and food derivatives have received a lot of attention as possible preventive and therapeutic agents [4, 5].

1.2 Vitamin E

1.2.1 Structures and forms

Vitamin E is a fat soluble molecule that consists of a phytyl tail and chroman head. Naturally occurring vitamin E is classified into tocopherols or tocotrienols based on the degree of saturation of the phytyl tail and abun-

dantly present in certain plant products such as palm oil, cereal grains, wheat germ and rice bran. Tocopherols and tocotrienols are further classified into sub-groups α, β, γ and δ depending on the position of the methyl group on the chroman head. Furthermore, the synthetic form of vitamin E, (all-rac-α-tocopherol) and derivatives of vitamin including RRR-α-tocopheryl succinate (VES) and RRR-α-tocopheryloxyacetic acid also called RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA) have been investigated for anticancer properties but they differ from natural forms of vitamin E in their structure and function [6].

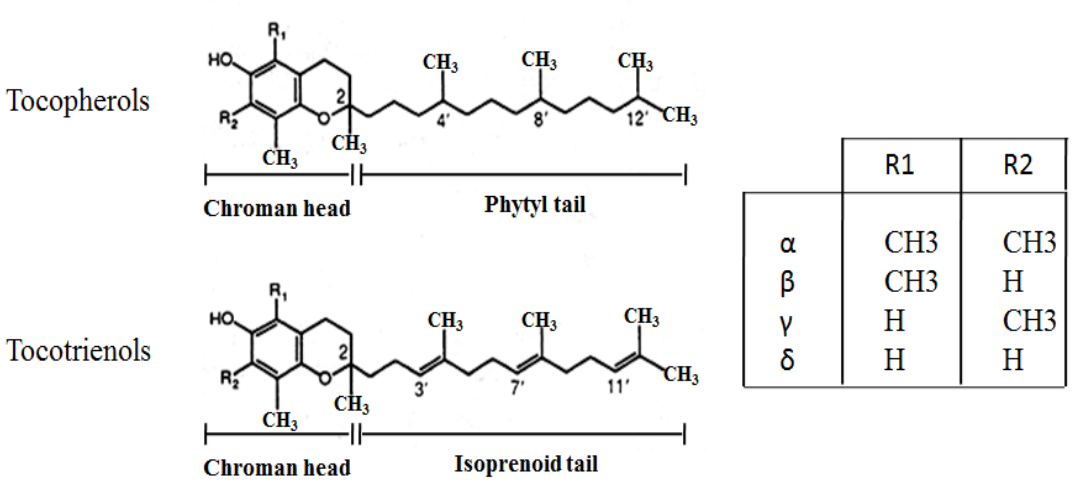


Figure 1.1: Structure of natural source forms of vitamin E.

Natural source vitamin E form are classified as tocopherols or tocotrienols based on presence of phytyl tail or isoprenoid tail, respectively. Position of methyl groups in the chroman head determine the sub-type of tocopherol or tocotrienol.

1.2.2 Absorption and metabolism

As vitamin E is lipid soluble, its absorption from food or supplements are dependent on general lipid absorption processes. Vitamin E in food is emulsified and incorporated into chylomicrons along with other lipids in intestinal enterocytes and enters the vascular system via the lymphatic system. Lipoprotein lipases (LPL) expressed on the surface of many tissues lead to triglyceride lipolysis delivering vitamin E to the respective tissues along with other lipids. The chylomicron remnants are absorbed by the hepatic tissues where they are repackaged into very low density lipoproteins (VLDL) to be secreted again into the blood [7]. The transfer of vitamin E into VLDL is aided by -tocopherol transfer protein (α-TTP) which selectively enriches for RRR- α-tocopherol into the lipoproteins while eliminating the other forms of vitamin E via bile secretions [8]. Various forms of tocopherols exhibit differential binding ability with TTP and have been characterized as $\alpha > \beta > \gamma > \delta$-tocopherol [7]. Packaged vitamin E can be exchanged between VLDL, low density lipoprotein (LDL) and high density lipoprotein (HDL). α-TTP is critical for the transfer of vitamin E into lipoproteins for tissue distribution. Deficiency of α-TTP results in reduced absorption of α-tocopherol as demonstrated in α-TTP knockout (Ttpa-/-) mice fed synthetic all-rac-alpha-tocopherol [9]. In contrast, tocotrienols were well absorbed by Ttpa-/- mice and distributed to peripheral tissues even in the absence of α-TTP [9]. Excess vitamin E is eliminated from the body by metabolism and/or excretion [10]. The phytyl tail is subject to ω-oxidation by xenobiotic metabolizing enzymes, cytochrome P450 (CYP) enzymes, followed by several steps of β-oxidation resulting in the formation

of 2-carboxyethyl-6-hydroxychroman (CEHC) metabolites which accumulate mostly in the urine [8]. Furthermore, vitamin E itself has been observed to increase the activity of CYP enzymes, specifically CYP3A and CYP4F2 resulting in tight regulation of vitamin E concentrations [8].

1.2.3 Functions

Classically vitamin E has been identified as an antioxidant. It functions as a peroxyl radical scavenger protecting cellular membranes from lipid-derived free-radical damage [11]. The antioxidant property of vitamin E has been widely explored in a number of clinical trials. It has been observed that vitamin E supplementation can reduce the risk of chronic diseases such as cardiovascular disorder, artherosclerosis and neurodegenerative diseases [12–16]. They have also been identified to possess antithrombotic and antiinflammatory properties but more unique biological functions have been associated with tocotrienols [17]. Tocotrienols have been characterized as displaying neuroprotective, antiangiogenic, anticancer and cholesterol lowering properties in addition to modulating immune functions [18] . Hence, gaining a better understanding of the functions and interactions of tocotrienols with other nutrients and drugs is important for development of effective therapeutics.

1.2.4 Anticancer properties of natural forms of vitamin E

Besides understanding the antioxidant properties of RRR- α-tocopherol, efforts have been made to determine chemopreventive roles. However this has been unsuccessful to date in pre-clinical animal models and human interven-

tion studies which have used tumor burden and reduction in cancer risk/ death respectively as end points. For example, the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study examined the effect of 50 mg of all-racemic-α-tocopheryl-acetate and 20 mg of β-carotene on lung and colorectal cancer in males [19]. Outcomes included a reduction in risk of colorectal cancer which was not statistically significant and no effect on lung cancer risk. However, a reduction in the rate of incidence and death of prostate cancer by 32% and 41%, respectively, was a major factor leading to the design of Selenium and Vitamin E Cancer Prevention Trial (SELECT) trial to determine the effect of vitamin E and selenium on prostate cancer. SELECT was a 12 year study in which male patients were provided dietary supplements consisting of 200 μg of selenium as L-selenomethionine or 400 IU of synthetic vitamin E as all-rac-α-tocopheryl acetate or a combination of both agents which was discontinued early due to lack of beneficial effects [20]. A recently concluded follow-up on the SELECT study reported a significant increase in prostate cancer risk in healthy men with vitamin E supplementation compared to healthy men in the placebo group [21]. A number of case-control and cohort studies have also examined the role of vitamin E as a chemopreventive agent but the literature is filled with mixed results from these studies and lack information about the form of vitamin E. The best example for case-control studies that provided reliable evidence for anticancer effects of vitamin E is CLUE I and CLUE II studies in which reduced serum concentration of γ-tocopherol was associated with increased risk for prostate cancer [22, 23].

At present, cell culture and animal studies demonstrate the importance

of defining the type of vitamin E studied in cancers and most of the evidence indicates that anticancer properties are limited to the γ- and δ-tocopherol, and tocotrienol forms of vitamin E individually or in mixtures enriched in these forms [6, 24].

1.2.4.1 Mechanisms of action of γ-tocopherol (γT)

Animal studies and cell culture studies have shown gamma-tocopherol to display more potent anticancer effects than alpha-tocopherol. γT has been shown to reduce tumor burden as well as lung and lymph node metastases in syngeneic BALB/c mouse 66cl-4-GFP mammary cancer model whereas RRR-αT was ineffective [25]. Similarly in a human breast cancer xenograft model of MDA-MB-231-GFP cells γT was observed to reduce tumor growth and it was associated with induction of apoptosis studied using TUNEL staining. γT has been seen to target multiple factors in the process of inducing apoptosis in various cancer cell lines [26]. In human breast cancer cells, γT induced apoptosis by enhancing death receptor DR5, inducing activation of caspases-8 and -9, reduction in protein levels of survival factors c-FLIP and Survivin [27]. In human prostate cancer γT induced apoptosis via interruption of the *de novo* sphingolipid synthesis pathway, release of cytochrome c, activation of caspases-9,-3 and -7 as well as caspase-independent pathways [28]. In human colon cells, γT induced apoptosis via activation of caspases-8, -3 and -7 but not caspase-9 as observed in human breast and prostate cancer cells [29]. A more complete understanding of the initiating and intermediate events will help us design more effective combination therapies.

1.2.4.2 Mechanisms of action of γ-tocotrienol (γT3)

The anticancer effects of tocotrienols have been demonstrated in many different animal models of cancer [24, 27–29]. Extensive work has also been carried out using *in vitro* systems to determine the mechanisms through which tocotrienols exert their anticancer effects [24]. Unlike tocopherols, only two human studies have been conducted to date to test the efficacy of tocotrienols in human subjects. The first study compared the accumulation of various forms of tocotrienols in the adipose tissue of patients with benign and malignant tumors consuming a diet rich in palm oil. It was observed that the concentration of tocotrienols was approximately 65% higher in the adipose tissues of patients with benign tumors compared to patients with malignant tumors [30]. In a recent pilot study conducted by the Malaysian Palm Oil Board, women with estrogen receptor positive primary tumors were provided a combination of tocotrienol and tamoxifen for a period of 5 years. Though the study did not achieve significance, it demonstrated a positive trend towards reduction of death due to breast cancer and recurrence compared to women provided only tamoxifen [31].

Tocotrienols similar to tocopherols and other natural agents have been reported to modulate multiple pathways during apoptosis. In human colon cancer cells, tocotrienols were observed to enhance TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis whereas in human breast cancer cells tocotrienols induced apoptosis via endoplasmic reticulum stress mediated activation of the death receptor-5 (DR5) pathway [32]. In various other cancer cells, tocotrienols activated the death receptors DR4 and 5 via reac-

tive oxygen species (ROS)/ Extracellular signal-regulated kinases (ERK)/p53 pathway [33]. Apoptosis induction also included Transforming growth factor beta (TGF- β)/ Fas/JNK signaling events, enhanced expression of proapoptotic Bax and Bid or downregulation of cyclin D1 and Bcl-2 [34, 35]. In addition to the above studies, tocotrienols have been characterized to induce cell cycle arrest, suppress transcription factors NF- κB and STAT-3, suppress hypoxia regulator - HIF-1 α, regulators of oxidative stress, i.e. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), angiogeneic factors and growth factor receptor signaling pathways [36–45]. Accumulating evidence demonstrates tocotrienols to be potent anticancer agents that can be combined with present therapies to enhance their efficacy and reduce toxicity associated side effects.

1.3 Simvastatin

1.3.1 Discovery and health effects

Simvastatin, a synthetic derivative of lovastatin, a fermentation product from the fungus *Aspergillus terreus* is a commonly used lipophilic cholesterol lowering drug. It targets the rate limiting enzyme - 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase (HMGCR) in the mevalonate pathway that ultimately reduces the overall circulating levels of cholesterol [46]. As a result, statin consumption reduces the risk of cardiovascular disorders. Inhibition of mevalonate synthesis also leads to inhibition of the synthesis of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) that are important intermediates in cell proliferation and survival [47]. The HMGCR enzyme is regulated at multiple levels (i.e. transcriptional, translational and

post-translational) by feedback from both isoprenoids and cholesterol in normal cells but in tumor cells the enzyme is more sensitive to the isoprenoid feedback [48, 49]. This varied response to sterol and non-sterol feedback maybe responsible for the anticancer effects of statins in tumor cells compared to immortalized but non-tumorigenic or normal cells [48, 49]. Simvastatin has been shown to exert anticancer effects in different types of cancer cells *in vitro* by inducing apoptosis and reducing tumor growth in pre-clinical xenograft models of human breast, colon and prostate [50–56]. Human epidemiological studies have demonstrated long-term use to be associated with reduction in breast cancer risk and recurrent disease [55, 56]. A number of factors have been identified to be modulated during induction of apoptosis in breast cancer including over-expression of Bax, reduced expression of Bcl-2, generation of reactive and nitric oxygen species, increased phosphorylation of JNK, suppression of AKT and nuclear factor kappa B pathway [57–61]. Simvastatin is also known to modulate the immune system as well as produce anti-inflammatory, anti-oxidant and anti-angiogeneic effects [62]. Due to these pleiotropic actions by simvastatin it has gained recognition as an anticancer agent yet its mechanisms of action need to be explored further to gain a better understanding of its preventive and therapeutic potential.

Despite the pleiotropic health benefits of statin, their application in cancer prevention and treatment is reduced by dose limited myotoxicity. Vitamin E, specifically tocotrienols have been shown to modulate the HMG-CoA reductase enzyme in the mevalonate pathway similar to simvastatin. Tocotrienol target HMG-CoA reductase enzyme both at the mRNA and protein level by

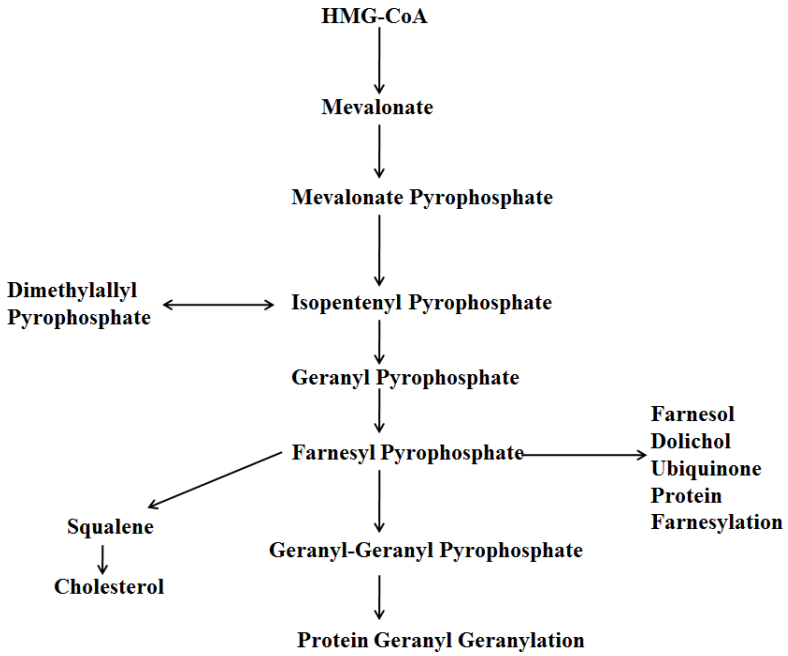


Figure 1.2: Mevalonate Pathway.

Products of the mevalonate pathway involve sterol (cholesterol), non-sterol (farnesol, dolichol, ubiquinone, farnesylated and genranylated proteins) and isoprenoid products (dimethylallyl pyrophosphate and isopentenyl purophosphate) which play important roles in cellular proliferation and survival. (Adapted from [49])

retaining sterol regulatory element binding proteins (SREBP) in the endoplasmic reticulum and proteosomal degradation of enzyme respectively [63]. Hence, in tocotrienol treatments negative feedback associated with lowered cholesterol leading to inhibition of enzyme degradation is ineffective unlike in statin treatments where lack of simultaneous enzyme degradation enables increase in HMGCR production both at mRNA and protein levels to compensate for statin induced reduction in enzyme activity [63]. Therefore, a combination

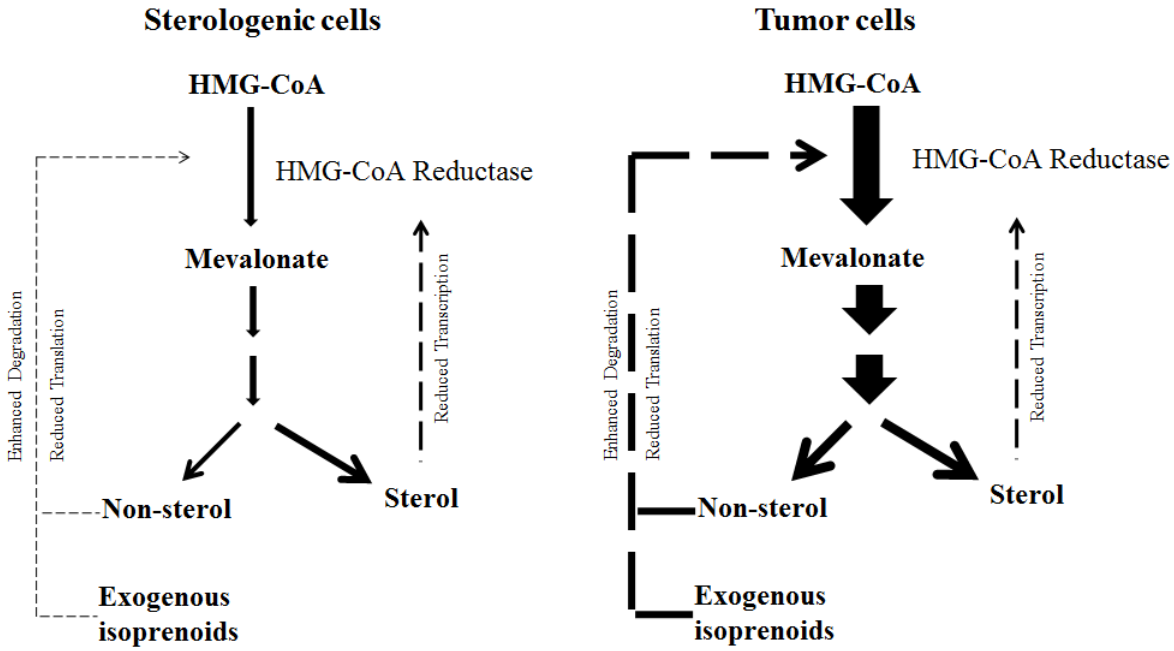


Figure 1.3: HMG-CoA Reductase feedback control.

The HMG-CoA reductase enzyme is subject to feedback control by sterol, non-sterol and isoprenoid products. In non-tumor cells, sterol feedback is the primary mechanism of control whereas in tumor cells, the elevated levels of HMG-CoA enzyme activity is resistant to sterol feedback and dependent on non-sterol and isoprenoids metabolites. (Adapted from [49])

of statin and tocotrienol has been tested for its combined ability to target the mevalonate pathway in cancer cells at low doses which limit dose associated toxicity [63]. The combination of γT3 and SVA has been shown to inhibit proliferation and cell cycle arrest in mouse mammary cancer cells. These effects have been attributed to suppression of MAPK, AKT and cyclin D1 protein expression levels [63]. The combination of simvastatin and γT3 show promise as a preventive and therapeutic combination for targeting breast cancer with

reduced toxic agents which needs to be further explored.

1.4 Apoptosis

Cells in the bodies can undergo four types of cell death, apoptosis, autophagy, cornification and necrosis [64]. Apoptosis is known as programmed cell death that is responsible for maintenance of cell populations during embryogenesis, development, aging and immune responses. Autophagy has opposing roles in the cell, i.e depending on the context of the stimulus it can activate cell survival or cell death. Necrosis is a toxic form of cell death accompanied by inflammatory responses [65, 66]. Unlike necrosis, apoptosis is a controlled, energy dependent process that does not result in non-specific inflammatory responses. Apoptosis is identified by specific morphologic changes such as cell shrinkage, condensation of cellular contents, DNA fragmentation, membrane blebbing and formation of distinct apoptotic vesicles [65, 66]. There are two distinct pathways of apoptosis that can cross-talk with each other: namely, extrinsic or death receptor mediated pathway or intrinsic mitochondria pathway [67–69]. Both these pathways involve activation of a series of cysteine-dependent aspartate-directed proteases (caspases) that amplify cellular stress or cell surface receptor activation leading to potent cell death. These enzymes are usually expressed in inactive forms and require recruitment to adaptor proteins followed by cleavage of the inhibitory prodomains to be activated [68]. Caspases are organized in a hierarchy as initiator and executioner caspases. When a cell receives an apoptotic trigger, the initiator caspases-2, -8, -9, -10 are activated by cleavage which in turn lead to activation

of effector caspases-3, -6, -7 [70]. Cellular stresses such as radiation, hypoxia and toxins or absence of growth factors, hormones or cytokines may stimulate the intrinsic pathway which involves changes in the mitochondrial membrane permeability and release of two different classes of proteins [65]. The first group of proteins includes cytochrome c, second mitochondrial derived activator of caspase/ direct inhibitors of apoptosis (IAP)-Binding protein with a low pI (Smac/Diablo) and serine protease HtrA2/Omi [71]. Cytochrome c forms an apoptosome with Apaf-1 and pro-caspase-9 which activates caspase-9 further leading to activation of caspase-3 [72]. Smac/Diablo and HtrA2/Omi amplify apoptosis by inhibiting activity of inhibitors of apoptosis proteins [73]. The second group of proteins includes apoptosis inducing factor (AIF), endonuclease G (EndoG) and caspase-activated-DNase (CAD) [74]. AIF and EndoG induce DNA fragmentation in a caspase-independent manner whereas CAD cleaves nuclear DNA following activation by caspase-3 [65, 75, 76]. The permeability and membrane potential of the mitochondria is controlled and regulated by members of the Bcl-2 family of proteins [65, 66]. These proteins are classified as anti- or pro-apoptotic depending on their function. The ratio of these anti- versus pro-apoptotic members in mitochondria determines cell fate: survival versus death response to a stimulus. The extrinsic pathway requires binding of ligands such as Fas ligand (FasL), or TNF-related apoptosis-inducing ligand (TRAIL) to death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily [77, 78]. All TNF receptors contain a death domain in the cytoplasmic region that transmits the death signal from the engaged receptor to the cytoplasmic adaptor proteins [79], a

adaptor proteins Fas-associated death domain (FADD) or tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD). FADD and receptor-interacting protein (RIP) are recruited via their death domains to the activated receptor that in turn recruits initiator caspase-8 in its inactive form. This complex is known as the death-inducing signaling complex (DISC) and results in autocatalytic cleavage of caspase-8 [79]. Executioner caspase-3 is activated by caspase-8, triggering apoptosis. Similar to the intrinsic pathway, anti-apoptotic factors also control the response of the cell to a death receptor initiated signal. Cellular FLICE inhibitory protein (c-FLIP) is similar in structure to caspase-8 with a death domain but lacks catalytic activity. c-FLIP associates with FADD and pro-caspase-8 in DISC inhibiting the death signal [65, 66]. The intrinsic and extrinsic pathways of apoptosis can cross-talk to enhance the apoptotic cue. Activated caspase-8 cleaves the pro-apoptotic Bcl-2 homology 3 (BH3)-only protein, Bid, that translocates to the mitochondria as tBid where it can recruit other pro-apoptotic members of the Bcl-2 family of proteins, Bax and Bak to alter membrane potential and permeability inducing the intrinsic pathway [65, 66].

1.4.1 TRAIL, Death receptors, CHOP and JNK

Tumour necrosis factor apoptosis inducing ligand (TRAIL) is a trimeric transmembrane protein that recognizes death receptors DR4 (TRAILR-1), DR5 (TRICK-2/KILLER/TRAILR-2) as well as decoy receptors (TRAIL-R3/DcR1 and TRAIL-R3/DcR2) and antagonistic receptor osteoprotegerin/ OPG of the TNF family of receptors via the C-terminal conserved sequence

[78, 80]. TRAIL can be detected in vesicle-associated forms and in circulation when cleaved by cysteine proteases produced by activated monocytes and neutrophils. Commonly, interaction of TRAIL with DR4/5 receptors has been shown to induce apoptosis via recruitment of adaptor proteins FADD through the death domain (DD) and pro-caspase-8 via the death effector domain (DED) forming DISC through interaction of the death domains [79]. Activated capase-8 induces apoptosome formation activating caspase-9 and eventually effector caspases. In contrast, interaction of TRAIL with DcR1 or DcR2 does not lead to apoptosis due to their lack of death domain or presence of truncated death domains, respectively [78]. TRAIL mRNA and protein has been detected in many human tissues, yet normal tissues of the body are largely resistant to the apoptotic effects of TRAIL [68]. The differential response of normal and tumor tissues to TRAIL induced apoptosis is associated with the presence of decoy receptors in normal tissues that sequestered TRAIL away from the death receptors. TRAIL has been observed to be upregulated during infections in T-cells, dendritic cells, macrophages and natural killer (NK) cells [68]. It has also been observed to play a role in innate immunity. However, higher expression of death receptors DR4 and DR5 in transformed cells makes TRAIL an attractive anti-cancer agent that can specifically target tumor cells irrespective of their p53 status. Although, recombinant human TRAIL (rhTRAIL) showed promise in phase I trials, its short half-life in vivo as well as identification of TRAIL responsive receptors on non-transformed cells has raised concerns regarding its safety and efficacy [68, 77]. Nonetheless, more effective variants of TRAIL and agonistic antibodies against TRAIL-

receptors (DR4 and DR5) are being developed and tested in combination with chemotherapeutic agents or ionizing radiation in a number of phase I/II trials which show more potential than TRAIL alone. Despite high expression of death receptors on tumor cells, treatment with TRAIL has failed to induce apoptosis in certain tumor cells. A number of theories have been proposed for the observed resistance. The foremost being the over expression of decoy receptors DcR1/ DcR2 that changes the balance at the cell surface such that tumor cells remain non-responsive to TRAIL even in the presence of death receptors DR4 and DR5 [78]. In some cases, defective death receptors with dominant negative signal inhibition or increased loss/ shedding from cell surface which result in resistance to TRAIL [81]. Furthermore low levels of death receptors can contribute to resistance [82]. Apart from the death receptors and their ligands, resistance has also been attributed to regulators of the apoptotic processes. Alteration in caspase-8 activation, elevated expression of inhibitor of caspase-8, c-FLIP, inhibitors of apoptosis (IAPs), anti-apoptotic Bcl-2 family members as well as changes in p53 status have all been identified as mechanisms leading to TRAIL resistance [81, 83]. However, therapeutic agents that enhance death receptors expression and TRAIL responsiveness can be combined with TRAIL to overcome resistance and develop safer strategies [84]. Death receptor, DR5, can be upregulated by multiple transcription factors such as NF-kB, p53, CCAAT/enhancer binding protein homologous protein/ DNA damage gene 153 (CHOP/GADD153) and kinases that phosphorylate transcription factors such as JNK [85–90]. DR5 has been shown to be a direct downstream mediator of p53 induced apoptosis in cells with intact wild-type

p53 even in the absence of DNA damage. Forced expression of wild-type p53 in p53-null colon cancer cells enhanced DR5 expression levels [90]. NF-kB has been traditionally observed to upregulate anti-apoptotic factors; however, in human embryonic kidney cell line 293, it was shown that NF-kB in the presence of p53, cooperatively binds to the first intronic region in the DR5 gene promoter to enhance DR5 expression and subsequently induce apoptosis [89]. Pro-apoptotic factor JNK was seen to upregulate DR5 in a CHOP dependent manner wherein CHOP regulated DR5 expression by binding to 5-region of the DR5 gene [88]. Agents that regulate the above mentioned upstream mediators of DR5 may be useful in enhancing TRAIL responses when used in combination. CCAAT/enhancer binding protein homologous protein/ DNA damage gene 153 (CHOP/GADD153) is commonly upregulated during induction of endoplasmic reticulum stress (ER-stress) [91]. It is a transcription factor regulated by X-box-binding protein (XBP1), activating transcription factor 6 (ATF6) and activating transcription factor 4 (ATF4) that bind to cis-acting elements in the promoter region [92]. CHOP plays a critical role in the outcome of ER-stress either by reducing cell viability or inducing apoptosis based on the extent of stress [93, 94]. Multiple genes are regulated by CHOP including ER oxidase (ERO1) and GADD34 both of which are involved in ER-stress responses a well as members of the Bcl-2 family of genes such as anti-apoptotic Bcl-2 and pro-apoptotic Bim [92, 95, 96]. c-Jun N-terminal protein kinase (JNK) belongs to the mitogen activated protein kinase (MAPK) superfamily [97]. Three isoforms of JNK have been identified, JNK1, 2 and 3 that arise due to alternate splicing and differential expression [98]. JNK1

and JNK2 are expressed in most cells whereas JNK3 is expressed specifically in brain and heart tissues [99]. Activated JNK can regulate transcription factors c-JUN, ATF-2, Elk-1, p53 and c-Myc as well as members of Bcl-2 family such as Bcl-2, Bcl-xL, Bim and BAD. JNK functions both as an anti- or pro-apoptotic factor and the role is defined by the source, strength and time period of the stimulus [99]. Hence, JNK plays an important role in survival, differentiation and apoptosis wherein the cellular context decides the outcome.

1.5 Sphingolipid pathway and ceramide

Sphigolipids are bioactive lipids consisting of a sphingoid backbone, long-chain fatty acid with an amide link and polar head groups [100]. Various types of sphingolipids have been identified such as ceramide, spingomyelin and glycosphingolipids, which act as second messengers to mediate cellular proliferation, survival and apoptosis in addition to providing structural support in the cellular membrane. Ceramide is a second messenger that plays an important role in growth, differentiation, and apoptosis [100, 101]. It is a major component of both cell surface and intracellular membranes. Ceramide is composed of sphingosine, a fatty acid and a hydroxyl head group [102]. Ceramide can be generated in mammalian cells by three different mechanisms: *de novo* synthesis, sphingomyelin hydrolysis and a salvage pathway [103]. The *de novo* synthesis of ceramide occurs in the endoplasmic reticulum by the condensation of precursors serine and palmitoyl-CoA to 3-ketosphinganine which undergoes further reduction, acylation and oxidation to eventually generate ceramide [104, 105]. In the sphingomyelin hydrolysis pathway, cermide is gen-

erated in the membranes by hydrolysis of sphingomyelin by sphingomyelinases (SMase). Multiple sphingomyelinases have been identified and are characterized by their pH of activity as neutral or acid SMases [106]. In the salvage pathway, glycosphingolipids such as glucosylceramide/lactosylceramide, sphingosine-1-phosphate or ceramide-1-phosphate are converted to ceramide at the plasma membrane by cerebrosidase, ceramide synthases and ceramide-1-phosphate phosphatase, respectively [103]. It has been observed that ceramide generated by SMases occur within minutes or hours of a stress stimulus whereas ceramide generated by the *de novo* pathway provides a sustained ceramide response [103]. Various stimuli initiate the *de novo* and SMase pathways such as oxidative stress, radiation and death receptors. Ceramide generated in the endoplasmic reticulum by the *de novo* synthesis pathway leads to ER-stress mediated apoptosis. Whereas ceramide generated by SMases causes increased ceramide accumulation in the membrane which leads to changes in the composition of membrane domains known as rafts [106]. Ceramide has been shown to associate with itself to form ceramide-enriched microdomains which can fuse to generate large ceramide-enriched macrodomains that amplify the primary death signal, thus triggering apoptosis [106]. Ceramide mediates apoptosis via suppression of protein kinase zeta-PKC (PKC), Ras, Raf-1, protein phosphatases PP1 and PP2A as well as by activation of JNK and induction of cathepsin D translocation [103]. Manipulation of the ceramide pathways have been considered for therapeutic purposes since it was observed that transformed cells are more sensitive to ceramide induced apoptosis compared to normal cells [102]. Hence, strategies that combine ceramide generating agents

with presently employed clinical agents may prove useful in enhancing therapeutic effectiveness while sparing surrounding normal cells.

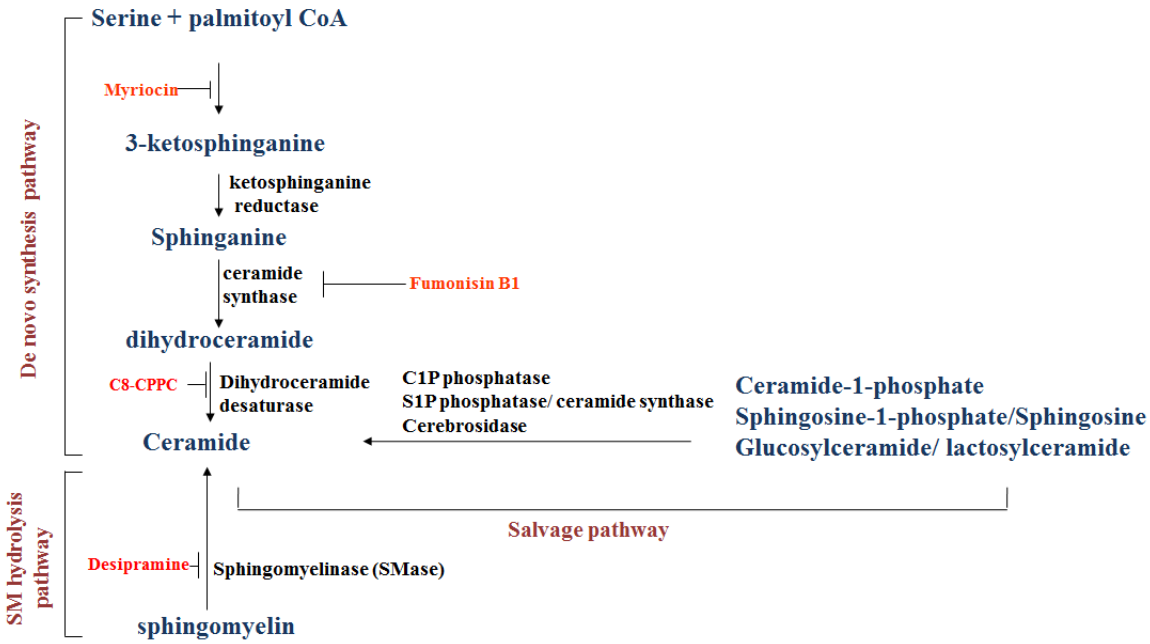


Figure 1.4: Ceramide synthesis pathways.

Myriocin, fumonisin B1, C8-cyclopropenylceramide (C8CPPC) and despiramine are well characterized chemical inhibitors of the enzymes involved in ceramide synthesis. Ceramide-1-phosphate phosphatase (C1P phosphatase); Sphingosine-1-phosphate phosphatase (S1P phosphatase). (Adapted from [103])

1.6 Cancer stem cells (CSCs) / Tumor initiating cells (TICs) and drug resistance

Based on the stem cell concept, CSCs / TICs represent a specialized small population of cells that exist within a tumor that are capable of differentiating into other cells of the tumor, which makes up bulk of the tumor. CSCs/

TICs in breast cancers have been characterized functionally as being able to initiate tumors in immune-comprised mice, demonstrate self-renewal measured in vivo as tumor formation in secondary mice using cells isolated from primary tumors or *in vitro* using mammosphere formation assays and differentiate into progeny that constitute the bulk of the tumor [107]. Operationally TICs have been characterized based on the expression of molecular markers distinct from the bulk tumor cells such as presence of CD44 (CD44+), absence of CD24 (CD24-), high aldehyde dehydrogenase activity (ALDH+), CD133+, PROCR+ and ESA+ [108–111]. Furthermore they display efficient DNA repair mechanisms making them more resistant to radiation or chemotherapy; epithelial to mesenchymal transition (EMT) leading to enhanced metastasis; and slow cycling time compared to bulk tumor cells identified by BrdU retention in pulse chase experiments [107, 111–114]. Recently it was observed that radiation and commonly used chemotherapeutic agents enhance stem cells populations *in vitro*, in animal models as well as in tumor tissues isolated from patients [115–118]. Using paired samples, tumor tissues from patients treated with neoadjuvant chemotherapy was shown to express higher percentages of TICs as measured by increase in CD44+/CD24- and ALDH+. Comparison of gene signatures derived from cells in primary mammospheres cultures with cells isolated from residual cancers or CD44+/CD24- enriched cells showed that these populations were enriched for TICs [114, 117, 118]. Several *in vitro* studies using established breast cancer cell lines treated with sublethal doses of radiation and chemotherapy provide evidence for resistance of these populations to common clinical therapies. Resistance has been associated with increased

expression of Wnt, Notch, Hedgehog, Her-2/PI3K/AKT, PI3K/mTOR/Stat3, DNA repair response proteins (CHK1, CHK2) or ATP-binding cassette (ABC) multidrug efflux transporters among others [107, 113, 119]. Hence, there is a need to develop therapeutic strategies targeting TICs to reduce recurrent disease and improve overall survival rates while ensuring normal cells remain uncompromised. A number of synthetic agents that target growth factor signaling and survival pathways are being tested for their ability to target TICs such as Her-2 monoclonal antibody (herceptin), EGFR/Her-2 inhibitor (lapatinib), BCR-ABL kinase inhibitor (imatinib) and telomerase inhibitors in clinics but there is also a need to develop less toxic therapies as anti-TIC therapies [113].

1.7 Stat3, stem cells and drug resistance

Signal transducers and activators of transcription (Stat) is a family of transcription factors that are activated by interferon receptors during cytokine signaling. At present seven Stat proteins have been identified which can be activated by cytokines (IFN α, IL-6, IL-12, IL-13) and growth factors (EGF, PDGF, insulin, IGF-1 and more) [120]. Stat3, a widely studied form of Stat in tumors, has been shown to be highly expressed in most malignant tissues compared to the non-malignant surrounding cells in breast cancer, prostate cancer, head and neck squamous cell carcinoma, multiple myeloma, lymphomas and leukemia, brain tumors, colon cancer, Ewing sarcoma, gastric cancer, esophageal cancer, ovarian cancer, nasopharyngeal cancer, and pancreatic cancer [121]. Stat3 signaling can be activated by oxidative stress,

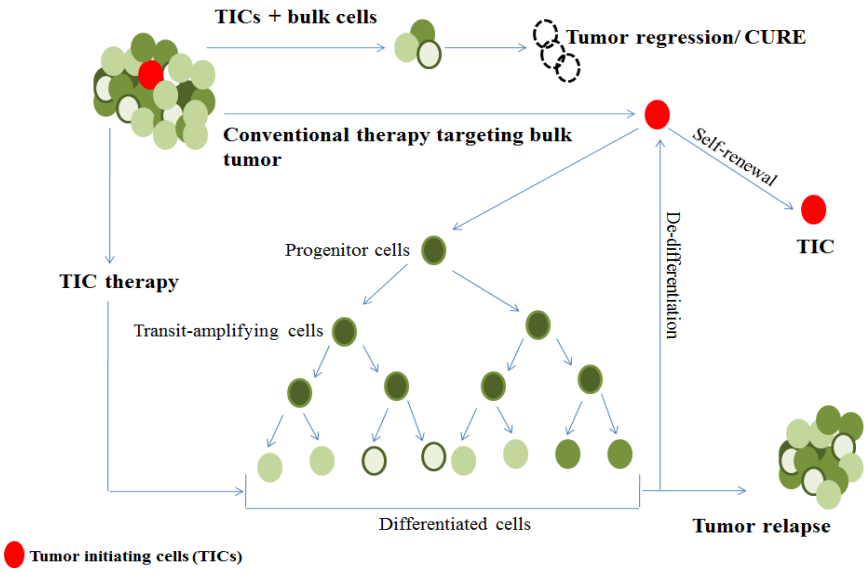


Figure 1.5: TIC vs Non-TIC treatment effects.

Conventional therapies target the non-TIC populations resulting in residual TICs that can differentiate to lead to treatment resistance and tumor relapse whereas treatments targeting both TIC and non-TIC would be more effective in providing cures. (Adapted from [119])

tobacco chewing, hepatitis C virus, ultraviolet B, lipopolysaccharide, osmotic shock, and progestins in addition to the cytokine and growth receptors [121]. Activation of Stat involves phosphorylation of the 705 tyrosine residue at the C-terminal domain which leads to formation of homo- or heterodimers that translocates to the nucleus and bind via DNA-binding domains upstream of the promoter region of the regulated genes [120]. Stat3 plays an important role in both developmental stages and progression of tumors via modulation of cell proliferation, apoptosis, angiogenesis, and immune system evasion. Well defined downstream mediators of Stat3 are Mcl-1, Bcl-xL and Survivin that act as anti-apoptotic factors; c-Myc and CyclinD1 that regulate cell proliferation;

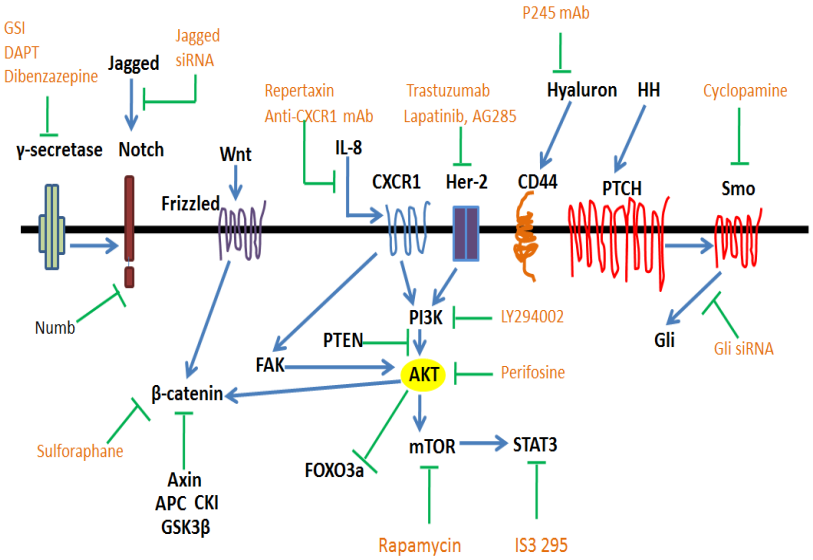


Figure 1.6: Anti-TIC treatments.

Ligands (blue arrows) bind to receptors activating TIC pathway that are important for maintenance of TICs. Chemical and DNA inhibitors (orange) that block activation of these pathways at various stages have been developed for clinical purposes. (Adapted from [113])

matrix metalloproteinase-9 (MMP-9) that mediates invasion; and vascular endothelial growth factor (VEGF) that regulates angiogenesis [120, 121]. Stat3 has been also been reported to be responsible for drug resistance and maintenance of stem cells [122, 123]. Hence, agents that target Stat3 may prove to be potent preventive and therapeutic agents to reduce resistance and recurrence, thereby improving survival outcomes.

1.8 Specific aims and objectives

Vitamin E and its antioxidant properties have been known for many years; but its role in prevention and treatment of chronic diseases including cancer is just coming to light. A review of the literature shows there is a large gap in the knowledge about vitamin E in general and about its anticancer potential in particular. Both γT and γT3 have been shown to induce apoptosis and reduce/ slow down tumor growth in different types of cancer both in syngeneic and xenograft animal models; inhibit proliferation but their clinical application is severely limited by their metabolism which reduces their *in vivo* availability. Moreover recently, γT3 has been shown to inhibit self-renewal of TICs and prevent tumor formation [124]. Work also shows that γT and γT3 regulate HMG-CoA reductase enzyme, the rate limiting enzyme in the mevalonate pathway. Statins have been prescribed for many years as cholesterol lowering drugs and target the mevalonate pathway. Though statins have also been demonstrated to display anticancer effects, not much is known about its mechanisms of actions. Hence, there is a need to improve our basic understanding of the mechanisms of action of naturally occurring forms of vitamin E and the commonly used clinical agent, statins such that they might be combined to yield better efficacy and fewer toxic side effects in prevention and treatment of cancer. Studies in this dissertation are focused on the following aspects: 1) To identify the anticancer mechanisms of simvastatin, a commonly prescribed cholesterol lowering drug; 2) To obtain a more complete understanding of the anticancer mechanisms of γT that is commonly found in the US diet and identify the events common to both γT and γT3 induced apoptosis; 3) To identify

the contributing factors in drug resistance development and identify a possible therapeutic strategy to address drug resistance. This chapter 1 provided a brief review of the literature and background information on the various agents investigated. Chapter 2 describes the role of death receptor, DR5, and its upstream mediators in simvastatin-induced apoptosis and downregulation of antiapoptotic factors in human breast cancer cells. Chapter 3 is focused on gaining a better understanding of the upstream mediators of apoptosis in γT induced apoptosis. The effect of both γT and γT3 on *de novo* ceramide pathway will also be described. Chapter 4 determines key factors associated with drug resistance and addresses the contribution of Stat3 pathway to the same. Chapter 5 summarizes the present work and describes future directions for research in these areas.

Chapter 2

Simvastatin induces apoptosis of human breast cancer cells via activation of JNK/CHOP/DR5 and suppression of c-FLIP/Survivin

2.1 Abstract

Simvastatin-induction of apoptosis in a dose-dependent fashion in human breast cancer cell lines was shown to involve (i) enhanced expression of death receptor-5 (DR5), CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), and phosphorylated c-Jun N-terminal kinase (pJNK), and (ii) decreased levels of anti-apoptotic mediators c-FLIP (cellular-FADD-like IL-1alpha-converting enzyme inhibitory protein) and survivin. The necessary involvement of these pro-apoptotic and anti-apoptotic mediators was demonstrated by siRNA knockdown studies. siRNA knockdown of DR5, CHOP or JNK significantly decreased the ability of simvastatin to induce apoptosis and showed the necessity of a JNK/CHOP/DR5 amplification signaling pathway in simvastatin induced apoptosis. Moreover, siRNA knockdown studies showed that down-regulation of two key anti-apoptotic mediators, c-FLIP and survivin, was mediated by JNK/CHOP/DR5. Activation of JNK/CHOP/DR5 pro-apoptotic pathway and suppression of c-FLIP and survivin anti-apoptotic factors by simvastatin was shown to be dependent on simvastatin's known

property to block 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthesis. Exogeneous addition of either mevalonate or geranylgeranyl pyrophosphate (GGPP), but not farnesylpyrophosphate (FPP) or cholesterol inhibited simvastatin activation of JNK/CHOP/DR5 apoptotic pathway as well as inhibited down-regulation of anti-apoptotic mediators c-FLIP and survivin.

2.2 Introduction

Breast cancer incidence and death remains a major health concern for women [1]. Systemic treatments, including cytotoxic, hormonal, and immunotherapeutic agents, are effective initially in 90% of primary breast cancers and 50% of metastases; however, after variable periods of time drug resistance and toxicity limit treatment effectiveness [3, 125, 126], highlighting the need for new treatment regimens.

Statins are widely prescribed drugs used for the reduction of cholesterol levels via inhibition of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, the enzyme that catalyses the rate-limiting step in mevalonate synthesis. This pathway is critical for the cellular synthesis of cholesterol and its isoprenoid intermediates; such as, geranylgeranyl pyrophosphate (GGPP) and farnesylpyrophosphate (FPP). These mevalonate-derived prenyl groups exert pleiotropic effects on many essential cellular functions including cell proliferation, differentiation, and survival [127–129]. In vitro and preclinical studies as well as clinical studies have shown that various statins, particularly lipophilic statins, have antiproliferative, antiangiogenic, antimetastasis

and pro-apoptotic properties in many types of cancers including breast cancer [130, 131].

Simvastatin (SVA) is a commonly used lipophilic statin derived from lovastatin. SVA shows antitumor actions in vitro and in pre-clinical xenograft models of breast, colon and prostate as well as efficiacy as a chemopreventive agent in reducing the risk of breast cancer in humans with long-term use [50–55]. The ability of SVA to induce apoptosis in breast cancer cells is established and several insights into SVA's mechanisms-of-action in the induction of apoptosis have been gained [57–61]. Proposed mechanisms that contribute to SVA induced apoptosis in human breast cancer cells include: JNK phosphorylation [57], generation of reactive oxygen species [58], activation of inducible nitric oxygen species resulting in increase of nitric oxide [59], suppression of Akt [60] and suppression of NF-kB [61]. Despite this knowledge, a more complete understanding of SVA induced apoptosis is needed in order to improve SVA treatments by pairing SVA with agents that can act in an additive or synergistic manner. Here, for the first time, we show that SVA induces apoptosis in human breast cancer cells via activation of a death receptor DR5 pro-apoptotic pathway and suppression of c-FLIP and survivin, in a JNK/CHOP dependent manner which is subject to blockage by exogenously added mevalonate and geranylgeranylpyrophosphate.

2.3 Materials and Methods

2.3.1 Chemicals

Simvastatin sodium salt (SVA), an active form of simvastatin for in vivo use, was obtained from Calbiochem (San Diego, CA). Mevalonate (Me), geranylgeranyl pyrophosphate (GGPP) and farnesylpyrophosphate (FPP) were obtained from Sigma (St. Louis, MO).

2.3.2 Cell Culture

Human breast cancer cell lines; MDA-MB-231 [American Type Culture Collection (ATCC), Manassas, VA], MCF-7 (ATCC), SUM 159 (Asterand, Detroit, MI), acquired tamoxifen resistant MCF-7 (MCF-7/TamR, gift from Dr. Linda deGraffenried, University of Texas at Austin, Austin, TX), and acquired doxorubicin resistant MCF-7 (MCF-7/ADR, gift from Dr. Kapil Mehta, M.D. Anderson Cancer Center, Houston TX), human ovarian cancer cell line; A2780 (ATCC), human prostate cancer cell line; LNCap-GFP [LNCap cells stably transfected with green fluorescent protein (GFP), gift from Dr. LuZhe Sun, University of Texas Health Science Center at San Antonio, San Antonio, TX], immortalized non-cancerous human epithelia breast cell line; MCF-10A (ATCC) and human umbilical vein endothelial cells (HUVEC) (ATCC) were used in this study. SUM159 cells were cultured in Hams F12 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum, 5 μg/ml insulin, 1μg/ml hydrocortisone (Sigma-Aldrich, St Louis, MO), and 10 mM HEPES buffer. MCF-7/TamR and MCF-7/ADR cells were derived from the parental cell lines by selection with tamoxifen and doxorubicin [132, 133], respectively. MCF-

7/TamR cells were maintained in modified IMEM (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal stripped bovine serum (Invitrogen, Carlsbad, CA), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 × MEM non-essential amino acid solution (Sigma-Aldrich, St Louis, MO), 2 × MEM vitamins solution (Sigma-Aldrich, St Louis, MO) and 100 nM tamoxifen (Calbiochem, Gibbstown, NJ). MCF-7/ADR cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and selected every three passages with 1g/mL of doxorubicin. HUVEC were cultured in endothelial cell basal medium with growth supplements (EGM-2 Bullet Kit, Clonetics). MCF-10A, A2780, and LNCap cells were cultured as described previously by Yu W et al., [134], Jia L et al., [135] and Sun M et al., [136], respectively. To mimic the non-serum condition of tumors, studies were conducted with 2% serum. Cells were plated at 3 x 105 cells/100 mm dish or 6 x 105/150 mm dish for apoptosis and western blot analyses, respectively. Cells were allowed to attach overnight before treatments. SVA dissolved in ethanol at 20 mM was used as stock solution. Equivalent levels of ethanol were used as vehicle control (VEH) for experiments.

2.3.3 Quantification of apoptosis

Apoptosis was quantified using an Annexin V-FITC/PI assay (Indra Mahajan, Bratton Lab, UT-Austin, TX) as described previously [32, 137]. Annexin V-FITC/PI assay measures the amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to

membranes of apoptotic cells) and amount of propidium iodide (PI), a dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in the late stages of apoptosis and binds DNA. Fluorescence was measured using Fluorescence Activated Cell Sorter (FACS) analyses with a FACSCalibur flow cytometer and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA). Cells displaying phosphatidylserine on their surface (positive for annexin-V fluorescence) were considered to be apoptotic.

2.3.4 Western blot analyses

Preparation of whole cell protein extracts and western blot analyses were conducted as described previously [32, 137]. Protein bands were imaged and quantified after correcting for GAPDH loading control using Scion Image Software (Scion Corporation, Frederick, MD). Primary antibodies used in this study were; pJNK, total JNK, CHOP, c-FLIP and survivin (Santa Cruz Biotechnology, Santa Cruz, CA), DR5, Caspase-8 and Caspase-9 (Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson Immunoresearch, Rockford, IL).

2.3.5 Small interfering RNA (siRNA) transfection

A scrambled RNA duplex that does not target any known genes was used as the nonspecific negative control for RNAi (referred to as control

siRNA). Transfection of siRNAs to JNK2/1, CHOP, DR5 and Itch or control (Ambion, Austin, TX) was performed in 100 mm^2 cell culture dishes at a density of 2×10^6 cells/dish using Lipofectamine-2000 and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. One day after transfection, the cells were re-cultured in 100 mm^2 dishes at 5×10^5 cells/dish and incubated for 1 day followed by treatments.

2.3.6 Statistical Analyses

The students t-test was used to determine statistical differences between treatment and control values. Differences were considered statistical significant at $p < 0.05$.

2.4 Results

2.4.1 SVA induces human breast cancer cells to undergo apoptosis

SVA treatment of MDA-MB-231 and MCF 7 cells for 3 days at 0.625, 1.25 and 2.5, and 1.25, 2.5 and 5 μM, respectively, significantly induced apoptosis in both cell lines in a dose response manner (Fig 2.1 A & B) where doses up to 2μM are achievable in humans [138].

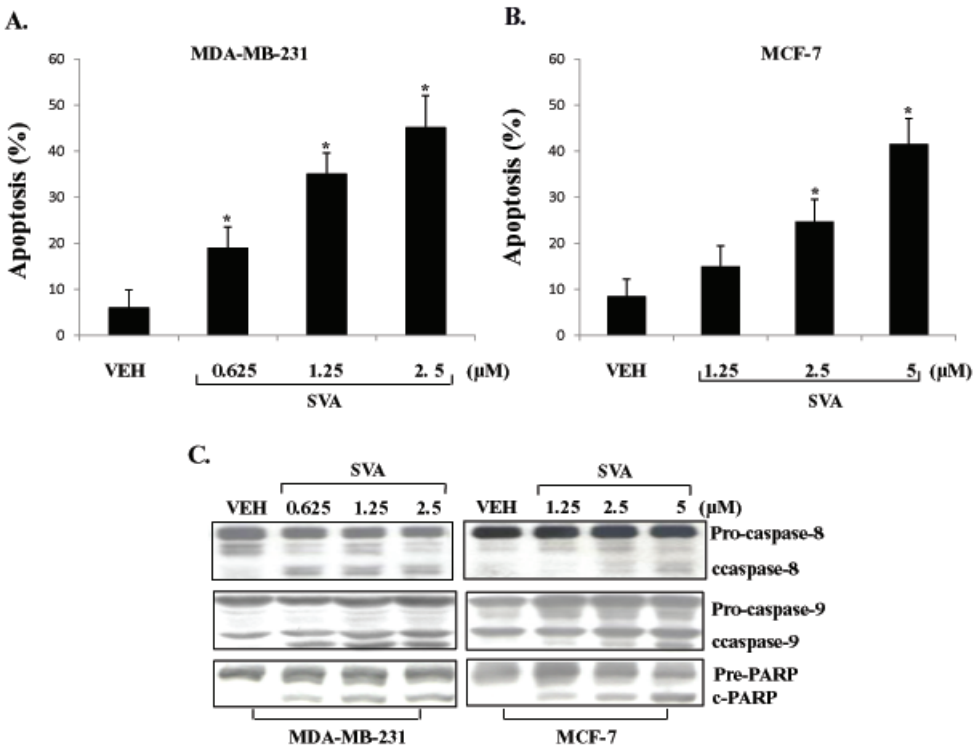


Figure 2.1: SVA induced dose-dependent apoptosis in MDA-MB-231 and MCF-7 human breast cancer cells. MDA-MB-231 and MCF-7 cells were treated with SVA for 3 days with 0.625, 1.25, and 2.5 and 1.25, 2.5 and 5.0 μM, respectively. Apoptosis was determined by Annexin V/FACS analyses (A & B). Western blot analyses were carried out to detect cleavage of PARP, caspase-8 and caspase-9 (C). A and B data are depicted as mean $\pm$ S.D. of three independent experiments. C data are representative of two or more independent experiments. *Significantly increased in comparison to vehicle (VEH), $P < 0.05$.

Based on EC50 values, the sensitivity of breast cancer cell lines to SVA induced apoptosis was shown to differ. MDA-MB-231 cells with an EC50 value of 2.5 were more sensitive to SVA-induced apoptosis than MCF-7 or SUM 159 breast cancer cell lines with EC50 values of 5.0 and 3.3, respectively (Table 2.1). Unexpectedly, data showed MCF-7/TamR and MCF-7/ADR resistant

cell lines to be more sensitive to SVA induced apoptosis than MCF-7 cells, with EC50 values of 1.5 and 1.6 μM, respectively (Table 2.1). These data suggest that SVA can be used as a treatment for chemotherapy resistant breast cancers by itself or in combination with other agents which will lower the effective doses of SVA required for treatment. Furthermore, data show that MCF-10A, an immortalized non-cancerous human epithelial breast cell line, is more resistant to SVA with EC-50 value $> 20\mu M$ (Table 2.1), suggesting that SVA's apoptotic property is not observed in non-cancerous human epithelial breast cells. Western blot analyses of whole cell extracts from SVA-treated MDA-MB-231 and MCF-7 cells show SVA to induce dose-dependent PARP cleavage, a marker for caspase dependent apoptosis, and caspase-8 and -9 cleavage, biochemical indicators of caspase activation (Fig 2.1C). These data further confirm the pro-apoptotic properties of SVA, and show that SVA induced apoptosis is associated with caspase-8 activation, suggesting involvement of death receptor-mediated apoptotic pathways.

2.4.2 SVA-induced apoptosis is associated with enhanced DR5 protein expression

MDA-MB-231 and MCF-7 cells treated with different levels of SVA for 3 days showed increased levels of DR5 (L/S) protein (Fig 2.2 A) in comparison to vehicle control. We tested the generality of SVA in MCF-7/ADR, SUM 159 and MCF-7/TamR cell lines and showed that treatment with 0.625 μM SVA for 3 days enhanced DR5 (L/S) protein levels in all three cell lines (Fig 2.2 B).

Cell Types	EC_{50} of apoptosis (μM)
MDA-MB-231	2.72 ± 0.21
MCF-7	6 ± 1
MCF-7/TamR	0.8 ± 0.2
MCF-7/ADR	0.76 ± 0.25
SUM159	4 ± 0.62
MCF-10A	> 20

Table 2.1: Determine sensitivity of breast cancer cell lines to SVA-induced apoptosis.Breast cancer cell lines and immortalized non-cancerous human epithelia breast cell line MCF-10A were treated with 0.625, 1.25, 2.5, 5, 10 and 20 μM SVA for three days.EC_{50} apoptotic values for SVA treatment of breast cancer cell lines were determined by Annexin V/FACS analyses. Data are presented as mean ± S.D. of three individual experiments.

2.4.3 SVA up-regulates CHOP and pJNK

Since pJNK and CHOP has been shown to act upstream in the activation of DR5 (L/S) [21-22], studies were conducted to determine effects on SVA treatment on pJNK and CHOP protein expression. SVA treatment of MDA-MB-231 and MCF-7 breast cancer cells for three days increased pJNK and CHOP protein expression in both cell lines in a dose-dependent manner in comparison with VEH (Fig 2.3A and B).

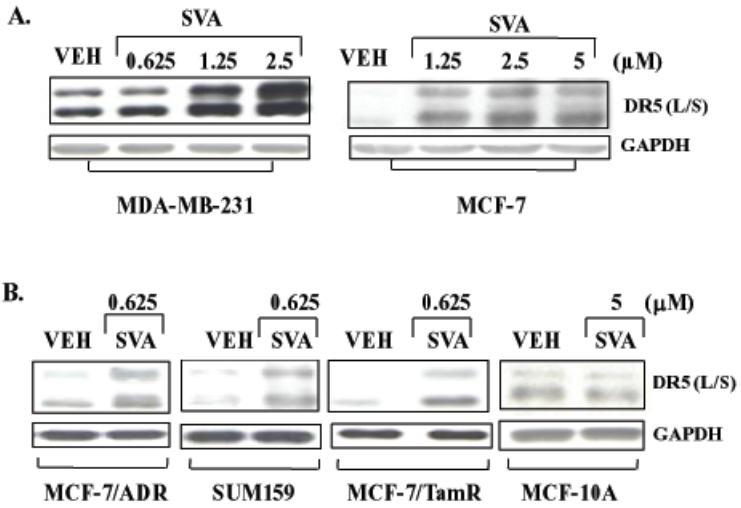


Figure 2.2: DR5 (L/S) is upregulated in SVA induced apoptosis. MDA-MB-231 and MCF-7 cells were cultured with three concentrations of SVA for 3 days (A). MCF-7/ADR, SUM159, and MCF-7/TamR cell lines were cultured with 0.625 μM SVA for 3 days (B). Western blot analyses were performed to evaluate DR5 (L/S) protein levels (A and B). A and B data are representative of two or more independent experiments.

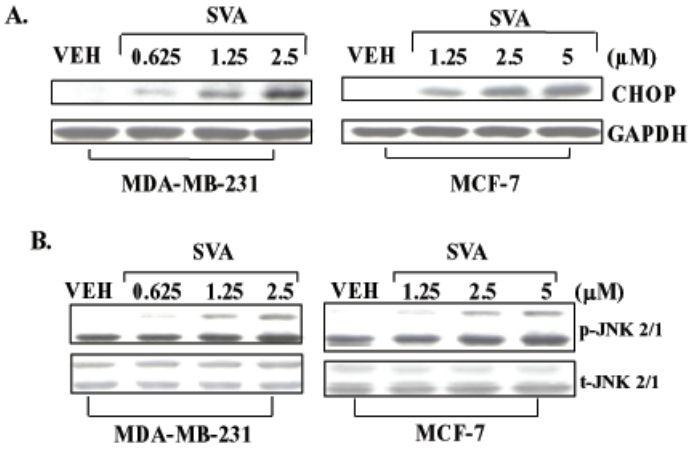


Figure 2.3: CHOP and pJNK is upregulated in SVA induced apoptosis.MDA-MB-231 and MCF-7 cells were treated with 0.625, 1.25 and 2.5, 1.25, 2.5 and 5 μM, SVA, respectively, for 3 days. Western blot analyses were performed to evaluate CHOP protein levels (A)and pJNK 2/1 and total JNK (t-JNK2/1) protein levels (B). A and B data are representative of two or more independent experiments.

2.4.4 SVA up-regulation of CHOP and DR5(L/S) and apoptosis is JNK2 dependent

siRNA to JNK2, CHOP and DR5 significantly reduced the ability of SVA to induce apoptosis in both cell lines as measured by Annexin V (Fig 2.4A) and PARP cleavage analyses (Fig 2.4B). siRNA to JNK2, CHOP and DR5 significantly reduced the ability of SVA to induce apoptosis in MDA-MB-231 and MCF-7 cell lines in comparison to control siRNA (Fig 2.2 C), and reduced the ability of SVA to induce apoptosis as measured by cleavage of PARP, and reduced the ability of SVA to increase pJNK, CHOP and DR5 (L/S) protein levels (Fig 2.2B). These data confirm that JNK2 is involved in SVA induced apoptosis.

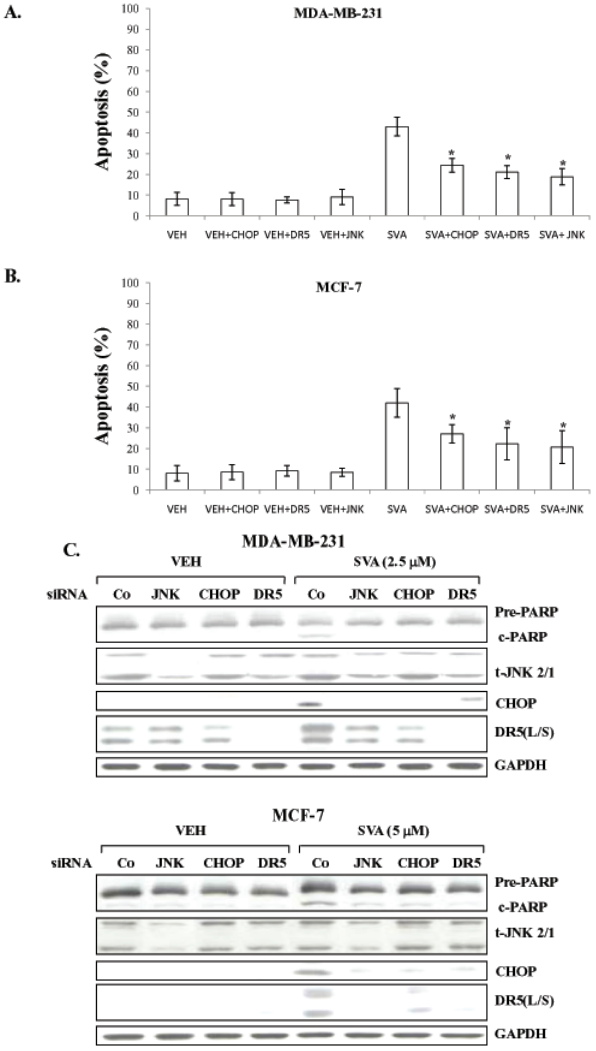


Figure 2.4: SVA induced increase in CHOP and DR5 proteins and apoptosis are JNK dependent.MDA-MB-231 and MCF-7 cells were transiently transfected with siRNA to JNK, CHOP and DR5 using non-specific siRNA as negative control followed by treatment with 2.5 and 5 μM SVA respectively for 3 days. Apoptosis was determined by Annexin V/FACS analyses (A and B). Western blot analyses were performed to evaluate PARP cleavage, pJNK 2/1, CHOP, and DR5 (L/S) protein levels using GAPDH as loading control in both cell lines (C). C data are representative of two or more independent experiments. A and B data are presented as the mean $\pm$ S.D. of three independent experiments. *significantly reduced in comparison to cells cultured with SVA plus control siRNA, $P < 0.05$.

Furthermore, siRNA to JNK blocked the ability of SVA to increase CHOP and DR5 (L/S) protein levels(Fig 2.4C). These data show that SVA induced increased CHOP and DR5 protein levels are down stream of JNK2/1.

2.4.5 SVA decreased levels of anti-apoptotic factors c-FLIP (L) and survivin are regulated by JNK/CHOP

Treatment of MDA-MB-231 and MCF-7 cells with different levels of SVA for 3 days reduced c-FLIP (L) and survivin protein levels in a dose-dependent manner with GAPDH as loading control (Fig 2.5A, top panels 1 & 2). c-FLIP mRNA was not markedly regulated by SVA (Fig 2.5A, panel 4). Survivin mRNA levels were downregulated by SVA only at the higher concentrations of SVA (Fig 2.5A, panel 5). These data suggest that SVA downregulation of c-FLIP is mainly regulated at the protein level while downregulation of survivin may involve both transcriptional and post-transcriptional processes. Furthermore, siRNA knockdown of JNK or CHOP prevented the ability of SVA to reduce c-FLIP (L) and survivin protein expression partially in both cell lines (Fig 2.5B).

2.4.6 SVA down-regulation of anti-apoptotic factor c-FLIP (L) is integral to SVA mediated apoptosis

Since c-FLIP (L) plays an important role in inhibiting apoptosis, we hypothesized that SVA mediated downregulation of c-FLIP may play a critical role in SVA induced apoptotic events. Furthermore, c-FLIP (L) protein levels have been reported to be regulated by JNK via E3 ubiquitin ligase Itch [139]. Studies were carried out to determine if SVA mediated down-regulation of c-

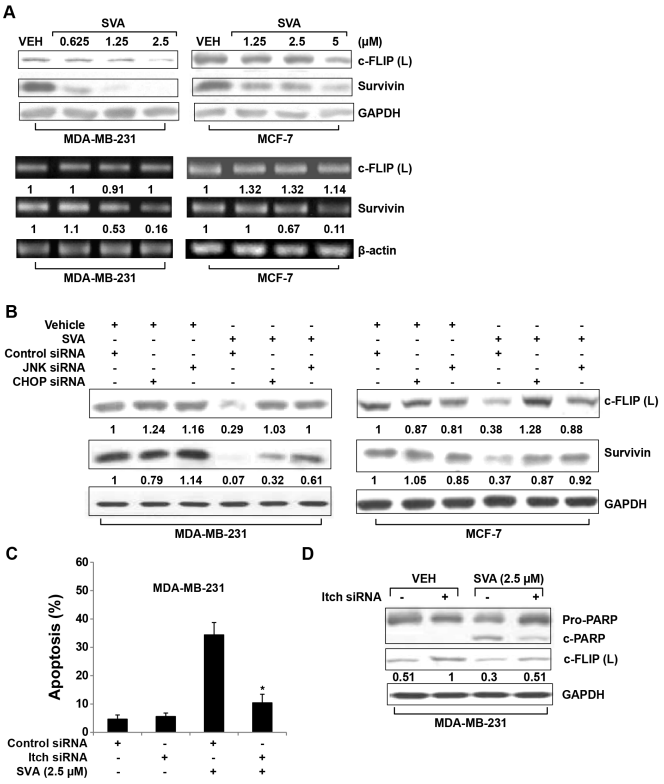


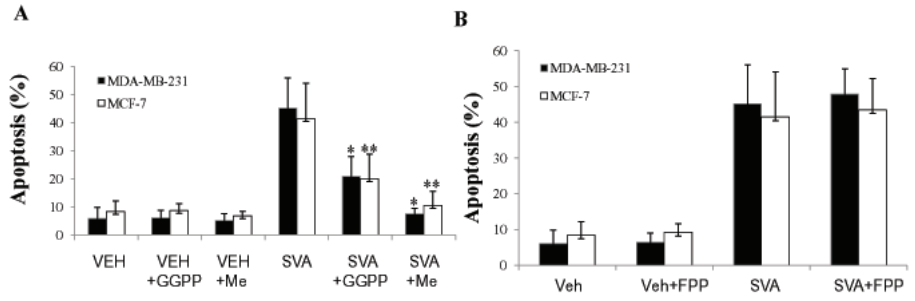
Figure 2.5: c-FLIP (L) and survivin are downregulated by SVA via JNK and CHOP dependent manner where JNK induced down-regulation of c-FLIP is mediated by Itch. MDA-MB-231 and MCF-7 cells were treated with three doses of SVA for 3 days. Western blot analyses were performed to evaluate c-FLIP (L) and survivin protein (A, panels 1 & 2) and mRNA levels (A, panels 4 & 5)). GAPDH and β-active served as load lane controls (A). MDA-MB-231 and MCF-7 cells were transiently transfected with JNK or CHOP siRNAs or control siRNA followed by treatment with 2.5 and 5 μM SVA, respectively for 3 days. c-FLIP (L) and survivin protein levels were determined by western blot (B). MDA-MB-231 cells transiently transfected with control or Itch siRNA were cultured with 2.5 μM SVA for 3 days. Apoptosis was determined by Annexin V/FACS analyses (C); western blot analyses were conducted to determine cleaved PARP and c-FLIP (L) protein levels using GAPDH as loading control (D). A, B and D data are representative of two or more independent experiments. C data are presented as the mean $\pm$ S.D. of three independent experiments. Numbers in A, B, and D data were generated by densitometric analyses *significantly reduced in comparison to cells cultured with SVA plus control siRNA, $P < 0.05$.

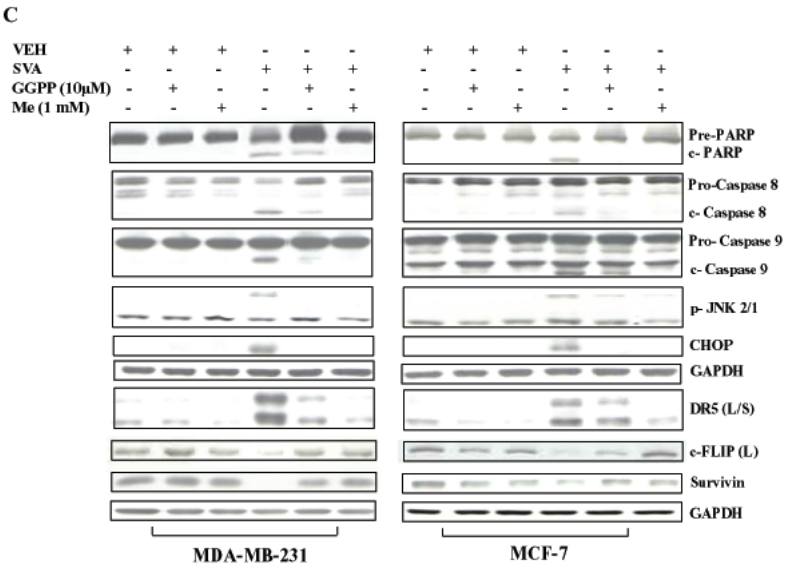
FLIP (L) was regulated by Itch and required for apoptosis. MDA-MB-231 cells transfected with scrambled control and Itch siRNA were treated with 2.5 μM SVA. siRNA knockdown of Itch significantly blocked SVA induced apoptosis as seen by Annexin V (Fig 5C) and cleaved-PARP analyses (Fig 2.5D), and SVA mediated downregulation of c-FLIP (L) protein levels (Fig 2.5D). These data indicate that SVA induced downregulation of c-FLIP (L) protein levels are regulated by Itch and involved in SVA mediated apoptosis.

2.4.7 SVA activation of JNK/CHOP/DR5 pro-apoptotic pathway and suppression of c-FLIP and survivin anti-apoptotic factors are blocked by addition of exogenous mevalonate or GGPP, but not FPP

To determine if SVA induced activation of JNK/CHOP/DR5 pro-apoptotic pathway and suppression of c-FLIP and survivin anti-apoptotic factors were regulated by SVA blockage of mevalonate pathway products namely, mevalonate (Me), GGPP or FPP, MDA-MB-231 and MCF-7 cells were cultured for two days with 10 μM GGPP or FPP or 1 mM Me with or without 2.5 and 5 μM SVA, respectively. Adding GGPP or Me significantly reduced the ability of SVA to induce apoptosis as determined by annexin V (Fig 2.6 A) and PARP cleavage analyses (Fig 2.6 C). Furthermore, both GGPP and Me blocked the ability of SVA to activate caspases 8 and 9, to up-regulate pJNK 2/1, CHOP and DR5 (L/S) protein levels and to down-regulate c-FLIP (L) and survivin (Fig 2.6 C). Treatment of MDA-MB-231 and MCF-7 cells with SVA in the presence of FPP had no effect on SVA induced events (Fig 2.6 B and D). In addition, exogenous cholesterol did not block SVA induced apoptosis (data not

shown). These data show that SVA-induced pro- and anti-apoptotic events are mediated via inhibition of mevalonate and GGPP and are independent of FPP and cholesterol.





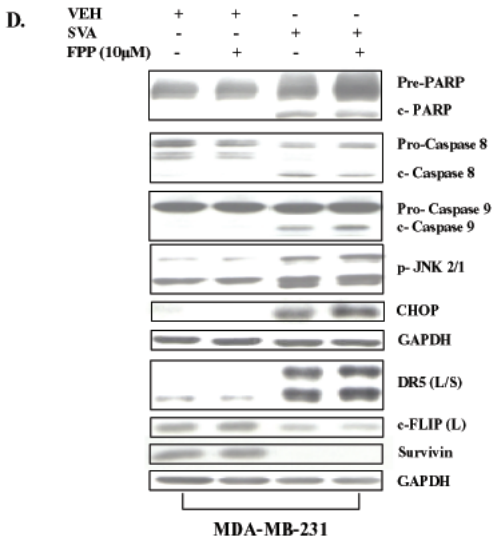


Figure 2.6: Exogenous mevalonate and GGPP (but not FPP) blocked SVA induced apoptosis, upregulation of JNK/CHOP/DR5 and downregulation of c-FLIP and survivin protein levels. MDA-MB-231 and MCF-7 cells were pre-treated with mevalonate (Me) (1 mM), GGPP (10 μM) or FPP (10 μM) for 2 hrs followed by treatment with 2.5 and 5 μM SVA, respectively for 3 days. Apoptosis was evaluated by annexin V assay (A and B). Cleaved PARP (c-PARP), casapses-8 and 9, pJNK 2/1, CHOP, DR5 (L/S), c-FLIP (L) and survivin protein levels were determined by western blot analyses (C, F). A and B data are presented as the mean ± S.D. of three independent experiments. * and ** significantly reduced in comparison to cells cultured with SVA only in MDA-MB-231 and MCF-7 cells respectively, $P < 0.05$. C and D data are representative of two or more independent experiments.

2.4.8 Simvastatin mediated apoptotic events are not limited to breast cancer cells

To determine if SVA mediated activation of DR5 and CHOP, and suppression of c-FLIP and Survivin are applicable to other cell types, human umbilical vein endothelial cells (HUVEC) (Fig 2.7A), A2780 human ovarian

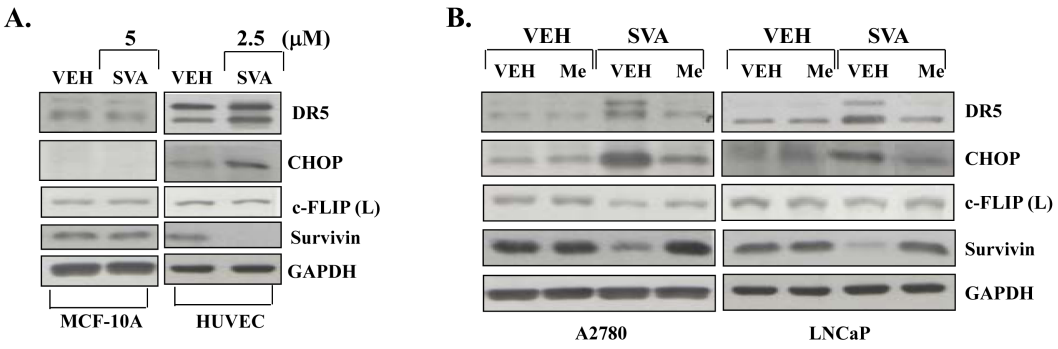


Figure 2.7: Effect of SVA treatment on MCF-10A, HUVEC, A2780 and LNCap cells. Immortalized non-cancerous human epithelia breast cell line, MCF-10A and human umbilical vein endothelial cells (HUVEC)(A), as well as A2780 human ovarian cancer cells and LNCaP human prostate cancer cells (B) were treated with SVA with or without mevalonate (Me) (1 mM) for 3 days followed by western blot analyses to determine the protein expressions of CHOP, DR5, c-FLIP and Survivin. Data are representative of two independent experiments.

cancer cells and LNCaP human prostate cancer cells (Fig 2.7B) were treated with SVA for three days with or without mevalonate. Data show that SVA induced upregulation of DR5 and CHOP protein expression, and downregulation of anti-apoptotic factors c-FLIP and Survivin protein expression are common to HUVEC and other cancer cells which can be reversed in the presence of mevalonate. Furthermore, treatment of immortalized non–cancerous human epithelia breast cell line, MCF-10A with SVA showed that SVA mediated events were restricted to non-cancerous breast cells.

Based on data presented in this paper, a diagram of signaling events involved in SVA induction of apoptosis of human breast cancer cell lines is provided in Figure 2.8. SVA blocks production of mevalonate and GGPP, resulting in activation of JNK2/1. pJNK2/1 downregulates anti-apoptotic

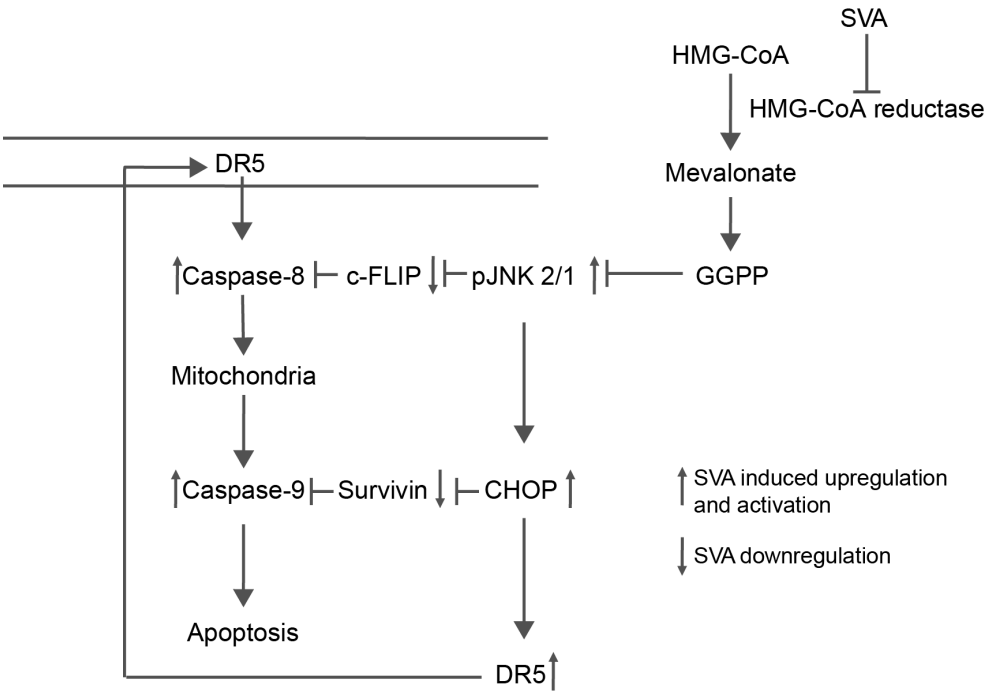


Figure 2.8: Proposed signaling pathways whereby SVA induces human breast cancer cells to undergo apoptosis. SVA inhibits mevalonate/GGPP pathway by blocking HMG-CoA reductase. This initial blockage leads to activation of JNK (pJNK2/1), pJNK2/1 induces increased levels of CHOP leading to DR5 amplification. pJNK2/1 inhibition of anti-apoptotic c-FLIP (L) promotes caspase-8 mediated pro-apoptotic cascade, and survivin down-regulation via CHOP enhances caspase-9 mediated apoptosis. These combined events lead to DR5 mediated caspase-8 cleavage and activation, leading to mitochondria/caspase-9 mediated apoptotic cascade.

factor c-FLIP and activate CHOP which produces both a decrease in anti-apoptotic factor survivin and increase in DR5 expression. The combination of decreased survival factors and increased DR5 produces increased tumor cell death.

2.5 Discussion

Although considerable progress has been made in understanding the anti-cancer actions of statins using tumor cells in culture and although in vivo anti-tumor efficacy of statins have been validated using xenograft models, important information about critical events in statin-induced apoptosis in cancer cells and effects on tumors remain unclear. Here, for the first time, studies demonstrate that simvastatin, a member of the statin family, induces apoptosis in human breast cancer cell lines via activation of DR5 death receptor events and suppression of anti-apoptotic factors c-FLIP and survivin via JNK/CHOP-dependent events which can be reversed by some but not all by-products of the mevalonate pathway for the biosynthesis of cholesterol. This new knowledge suggests that increases in protein levels of DR5 and CHOP which would be detectable by immunohistochemistry in tumor biopsied material may serve as useful biomarkers to assess patient tumor responsiveness to SVA treatment.

Novel findings in this paper are that SVA: (i) induces death receptor DR5 dependent apoptosis via up-regulation of DR5 protein expression (ii) induces up-regulation of CHOP protein levels via a JNK dependent event, and (iii) up-regulates DR5 and suppresses c-FLIP and survivin via JNK/CHOP-

dependent event. These data provide new insights into the anticancer actions of SVA, and provide information on potential biomarkers to be used in science-based decision making regarding the use of SVA in the treatment of cancer.

Data, for the first time, show that DR5 protein is up-regulated by SVA and involved in SVA induced apoptosis. Previous studies have shown that DR5 can be up-regulated by NF-kB [86, 87], p53 [87], JNK [32, 88, 137] and CHOP [32, 88, 137]. Data presented here show that SVA mediated up-regulation of DR5 is due at least in part to both CHOP and JNK. JNK has been reported to be activated by SVA and involved in SVA induced apoptosis of human breast cancer cells [57]. However, how JNK contributes to SVA induced apoptosis has not been addressed. Data presented here, for the first time, show that c-Jun N-terminal kinase (pJNK2/1) mediates SVA induced apoptosis via up-regulation of CHOP-dependent death receptor DR5 events. JNK has been reported to activate CHOP via an AP-1 binding site in the CHOP promoter region in HeLa cells [140]. A JNK/CHOP/DR5 signal transduction pathway has been reported to be activated and involved in apoptosis induced by several agents, including methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate [88], alpha-TEA [137] and gamma-tocotrienol [32]. Our data show that SVA induced apoptosis is also mediated by this JNK/CHOP/DR5 pathway.

Activation of JNK/CHOP/DR5 has been reported to be endoplasmic reticulum stress dependent [88, 140] and independent [141]. Our data suggest that SVA induction of JNK/CHOP/DR5 is endoplasmic reticulum stress independent because: (i) standard biomarkers of endoplasmic reticulum stress are not regulated by SVA, such as XBP-1 splicing, ATF-4 and pIeF2 protein ex-

pression (data not shown), and (ii) the endoplasmic reticulum stress inhibitor salubrinal did not block SVA induced apoptosis (data not shown).

Another important finding in this paper is that JNK not only contributes to up-regulation of CHOP and DR5, but also contributes to down-regulation of anti-apoptotic factors c-FLIP and survivin in SVA-induced apoptosis. c-FLIP, a protease-deficient caspase-8 homolog, inhibits death receptor mediated apoptosis via inhibition of caspase-8. Since our data show that SVA induces activation of caspase-8, the ability of SVA to down-regulate c-FLIP is proposed to be an important aspect of SVA induced apoptosis. Our data show that SVA suppression of c-FLIP is JNK dependent. JNK has been reported to degrade c-FLIP via phosphorylation and activation of the E3 ubiquitin ligase Itch pathway in TNF- and alpha-TEA induced cell death [137, 139]. Our data showing that siRNA to Itch recovered SVA-induced down-regulation of c-FLIP, demonstrated for the first time that JNK/Itch pathway is involved in degradation of c-FLIP, and is critical to SVA-induced apoptosis.

Down-regulation of survivin by SVA has been proposed to play an important role in SVA induced apoptosis. SVA has been reported to down-regulate survivin by suppression of Akt in human lung cancer A549 cells [60] and by suppression of NF-kB in human myeloid KBM-5, squamous cell carcinoma SCC4, and human embryonic kidney A293 cells [61]. Our data show that SVA at higher concentrations (1.25 and 2.5 M in MDA-MB-231 cells, and 2.5 and 5 M in MCF-7 cells) reduces levels of survivin mRNA, while SVA at lower concentrations (0.625 M in MDA-MB-231 cells and 1.25 M in MCF-7 cells) down-regulates levels of protein but not mRNA, suggesting that

SVA may regulate survivin levels, at least in part at both the transcriptional and translational levels depending on SVA cellular uptake/retention. Furthermore, data reported here show that survivin is down-regulated by SVA in a JNK/CHOP-dependent manner. How JNK/CHOP regulates survivin is not known. Survivin has been shown to be regulated at the post-translation level in a cell cycle dependent manner with lower expression in the G1 phase and higher expression in the G2 phase [142]. Endoplasmic reticulum stress has been shown to down-regulate cyclin D1, leading to a G1 block [143, 144]. Similarly, CHOP has been shown to induce cell cycle arrest at G1/S check point [145]. Our data show that SVA downregulated cyclin D1 (data not shown) and published data show that SVA induced G1 block [146, 147]. Thus, one possibility is that JNK/CHOP contributes to the down-regulation of survivin in SVA induced cell death via CHOP mediated down-regulation of cyclin D1, leading to G1 block. Since SVA at high doses induces down-regulation of survivin mRNA, we cannot rule out the possibility that mechanisms other than G1 block may mediate down-regulation of survivin by SVA.

In agreement with other reports that SVA induced apoptosis can be inhibited by either exogenous mevalonate or GGPP, but not by exogenous farnesyl pyrophosphate (FPP) or by exogenous cholesterol [146], our data demonstrated that SVA up-regulation of JNK/CHOP/DR5 pro-apoptotic pathway and down-regulation of anti-apoptotic factors are dependent on lack of mevalonate and GGPP in human breast cancer cells. It has been shown that inhibition of mevalonate/GGPP reduces Rho signaling which contributes to JNK activation [148]. However, it is not at all clear how inhibition of Rho sig-

naling induces activation of JNK. In this regard, it has been reported that free radicals are generated in SVA induced apoptosis in human breast cancer cells via suppression of the GGPP/Rho pathway [57, 58]. Other studies have demonstrated that reactive oxygen species are capable of inducing ER-stress [149, 150], and to subsequently activate JNK by both ER-stress dependent [151] and independent mechanisms [152]. However, ROS activation of JNK via SVA has not been documented.

In agreement with the report from Kotamraju S and co-workers [59] our data also show that SVA does not induce apoptosis in noncancerous human epithelia breast cells. Furthermore, our data shows that SVA has no effect on pro-apoptotic markers CHOP and DR5, as well as anti-apoptotic markers c-FLIP and Survivin (Figure 7A) in MCF-10A cells, which confirms that SVA induces apoptosis in breast cancer cells, but not in noncancerous human epithelia breast cells. However, data show that SVA up-regulates protein levels of pro-death mediators CHOP and DR5 and down-regulates protein levels of anti-apoptotic mediators c-FLIP and Survivin in HUVECs (Figure 7A), suggesting that SVA may exert its pro-apoptotic effects on endothelial cells. Thus, it is of interest to further identify the pro-apoptotic effects of SVA on different types of non-cancerous cells. Since the growth of HUVEC's is very important for angiogenesis our finding may provide some insight into understanding SVA's actions targeting angiogenesis.

Furthermore, to determine the generality of SVA pro-apoptotic events, studies were conducted using human A2780 ovarian and human LNCaP prostate cancer cells to evaluate the effects of SVA on the protein expressions of CHOP,

DR5, c-FLIP and Survivin as well as the involvement of mevalonate pathway. Data show that SVA increased pro-apoptotic CHOP and DR5 protein levels and decreased anti-apoptotic c-FLIP and Survivin protein levels (Figure 7B). Exogenous mevalonate blocked SVAs ability to up-regulate CHOP and DR5 and down-regulate c-FLIP and Survivin (Figure 7B). These data demonstrate that SVA inhibition of the mevalonate pathway, leading to activation of CHOP/DR5 and suppression of c-FLIP and Survivin, is not breast cancer specific.

In conclusion, simvastatins dual anticancer actions of activation of pro-apoptotic and suppression of anti-apoptotic pathways in vitro contribute to its overall anticancer activity. In this study, we further documented key events that are involved in the anticancer actions of SVA via inhibition of Me/GGPP; namely, activation of phosphorylated JNK2/1/CHOP/DR5, and down-regulation of anti-apoptotic mediators c-FLIP (L) and survivin. Thus, the findings in this study provide important insights into the apoptotic mechanisms of action of SVA and information on biomarkers critical for SVA-mediated apoptosis which have potential to identify SVA-responsive patients as well as help with design of combination strategies to enhance the effectiveness of SVA and reduce toxicity.

Chapter 3

Targeting ceramide pathways in vitamin E induced apoptosis in human breast cancer cells

3.1 Abstract

This study examines mechanisms involved in the pro-apoptotic action of two vitamin E forms; gamma-tocopherol (γT) and gamma-tocotrienol (γT3), in human breast cancer cell lines. Previously, we reported that γT3 induces apoptosis via endoplasmic reticulum stress (ERS)-mediated c-Jun N-terminal kinase (JNK), CCAAT/enhancer binding protein homologous protein (CHOP), and death receptor-5 (DR5) pro-apoptotic pathway, and γT induces DR5 dependent apoptosis. Here, we report that γT induces elevated levels of ERS markers, phospho-JNK (pJNK), CHOP, and DR5 protein expression; as well as, decreases levels of anti-apoptotic cellular FLICE inhibitory protein (c-FLIP), B-cell lymphoma 2 (Bcl-2) and Survivin protein expression. siRNA knockdown of JNK, CHOP, or DR5 show that the ability of γT to induce apoptosis and suppress anti-apoptotic factors are JNK/CHOP/DR5 pathway dependent. Data also show that both γT and γT3 induce increased levels of cellular ceramides and dihydroceramides. Inhibition of *de novo* ceramide synthesis using chemical inhibitors blocked the ability of γT and γT3 to induce apoptosis, to activate JNK/CHOP/DR5 pro-apoptotic pathway, and

to suppress anti-apoptotic mediators c-FLIP (L), Bcl-2 and Survivin; thereby demonstrating the involvement of *de novo* ceramide synthesis in γT- and γT3-induced apoptosis. Taken together, our data showed that both γT and γT3 induce apoptosis via ceramide mediated ERS, upregulation of JNK/CHOP/DR5 pro-apoptotic signaling and downregulation of anti-apoptotic factors c-FLIP, Bcl-2 and Survivin.

3.2 Introduction

Breast cancer incidence and death rates remain a major health concern in the U.S. [1]. In general, systemic treatments, including chemotherapy, hormonal, and immunotherapeutic agents, are active at the beginning of therapy in 90% of primary breast cancers and 50% of metastases [2]. However, treatments are compromised due to initial or acquired drug resistance and toxic side effects [3, 125]. Therefore, the need for new regimens with low to no toxicity for breast cancer prevention and therapy remains. Ceramide, a lipid composed of sphingosine and a fatty acid, is one of the constituents of sphingomyelin that is a major component of mammalian cell surface and intracellular organelle membranes. Ceramide is generated in mammals by three independent processes: *de novo* synthesis, sphingomyelin hydrolysis and a salvage pathway [153, 154]. The *de novo* synthesis of ceramide occurs in the endoplasmic reticulum (ER) from precursors L-serine and palmitoyl-CoA via several intermediates [153, 154]. Ceramide generated in the ER becomes a component of membrane lipids such as; glycosphingolipids and sphingomyelin. The sphingomyelin hydrolysis pathway converts sphingomyelin to ceramide in cellu-

lar membranes via sphingomyelinase (SMase) by hydrolysis [153, 154]. In the salvage pathway, glucosylceramide/lactosylceramide, sphingosine-1-phosphate or ceramide-1-phosphate is converted to ceramide by cerebrosidase, ceramide synthases and ceramide-1-phosphate phosphatase, respectively [153, 154]. Ceramide is an important signaling molecule that regulates cellular growth, differentiation, and apoptosis [153, 154] and can be regulated by multiple stimuli, such as oxidative stress, radiation and death receptors [155–157]. Accumulation of ceramide in ER via *de novo* synthesis has been reported to induce ERS mediated apoptosis [154]. Whereas, activation of SMase by different stress signals or apoptotic signals [153–157] causes increase and accumulation of membrane ceramide to form ceramide-enriched lipid raft microdomains that can serve as platforms for apoptotic signal transmission or amplification [153, 154, 158]. Vitamin E consists of a group of structurally distinct naturally occurring compounds that are classified as tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ), and synthetic forms such as all-racemic-α-tocopherol (all-rac-αT; also referred to as dl-α-tocopherol); as well as, vitamin E analogs such as vitamin E sucinate (VES) and RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA) (reviewed in [6]). A subset of vitamin E compounds have been shown to be effective anticancer agents in vitro and in vivo, including γ- and δ-forms of tocopherol and tocotrienol (reviewed in [24, 159]). Accumulating evidence supports that γT, the most abundant form of vitamin E in the American diet, and γT3 obtained from palm oil, wheat germ, rice bran and barley, possess anticancer properties [24, 159]. Both γT and γT3 have been shown to inhibit tumor growth in a number of animal mod-

els and these anticancer effects are attributed to both induction of apoptosis and cell cycle arrest [24, 27, 32, 159]. γT and γT3 induced apoptosis has been reported in colon, prostate and breast cancer cell lines and involves activation of caspases -3, -7, -8 and /or -9 and release of cytochrome c [24, 27, 32, 159]. The extrinsic death receptor DR5 and alteration of sphingolipid pathways have been demonstrated to be necessary for γT- and γT3-induced apoptosis [27, 28, 32, 160]. Previously, we reported that γT3 induce DR5 dependent apoptosis via ERS mediated JNK and CHOP [32]. Here, for the first time, we demonstrate that γT also induces JNK/CHOP/DR5 dependent apoptosis and that it down-regulates key pro-survival/anti-apoptotic factors c-FLIP, Survivin, and Bcl-2. Furthermore, data show that *de novo* ceramide synthesis is activated and involved in both γT- and γT3-induced apoptotic events.

3.3 Materials and Methods

3.3.1 Chemicals

γT and γT3 were gift from TAMA Biochemical Company (LTD, Tokyo, Japan) and Malaysian Palm Oil Board (Kuala Lumpur, Malaysia), respectively. Myriocin (My), an inhibitor of serine palmitoyltransferase that is the first limited enzyme in *de novo* ceramide synthesis pathway, was obtained from BioMol (Plymouth Meeting, PA). C8-cyclopropenylceramide (C8CPPC), an inhibitor of the dihydroceramide desaturase enzyme, involved in the final step of the *de novo* ceramide synthesis pathway, was obtained from Matreya LLC (Pleasant Gap, PA).

3.3.2 Cell Culture

MCF-7 and MDA-MB-435 human breast cancer cell lines were cultured as previously described [14]. SUM159 human breast cancer cells (Asterand, Inc. Detroit, MI) were cultured in Hams F12 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS), 5 μg/ml insulin, 1μg/ml hydrocortisone (Sigma-Aldrich, St Louis, MO), and 10 mM HEPES buffer. Acquired adriamycin/doxorubicin (AD) resistant MCF-7 (MCF-7/ADR; obtained from Dr. Kapil Mehta, M.D. Anderson Cancer Centre, Houston TX) and Acquired tamoxifen (Tam) resistant MCF-7 (MCF-7/TamR; obtained from Dr. Linda deGraffenried, University of Texas at Austin, Austin, TX) human breast cancer cell lines were derived by selection from parental cells cultured in the presence of doxorubicin or tamoxifen, respectively and cultured in the conditions described previously [132, 133]. For experiments, FBS was reduced to 2%. γT and γT3 were dissolved in 1:4 DMSO/ethanol at 40 mM as stock solution. Equivalent levels of 1:4 DMSO/ethanol was used as vehicle control (VEH).

3.3.3 Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturers instructions (Invitrogen, Carlsbad, CA) and published procedure [32]. Annexin V/FITC was a gift from (Dr. Shawn Bratton, University of Texas at Austin).

3.3.4 Western blot analyses

Western blot analyses were conducted as described previously [32]. Primary antibodies to the following proteins were used in this study; PARP, pJNK, total JNK, GRP-78, CHOP, cFLIP, Bcl-2 and Survivin (Santa Cruz Biotechnology, Santa Cruz, CA), DR5 (Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson Immunoresearch, Rockford, IL); and bovine-anti-goat (Santa Cruz Biotechnology).

3.3.5 Small interfering RNA (siRNA) transfections

A scrambled RNA duplex that does not target any known genes was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of siRNAs to JNK1/2, CHOP, DR5 or control (Ambion, Austin, TX) was performed as described previously [27, 32].

3.3.6 Lipid extraction and measurement of sphingolipids using LC-MS/MS

Lipid was extracted according to the method published before [133]. Briefly, cell pellets were resuspended in 500 μL of methanol, 250 μL of chloroform and 50 μL of water after the addition of 20 μL of internal standard mixture (Avanti Polar Lipids, Alabaster, AL). The suspension was tip sonicated for 20 sec and then incubated overnight at 48 ^oC. 100 μL of the suspension was taken to determine total choline-containing phospholipids as previously

described [28], and the rest was added with 75 μL of 1M potassium hydroxide in methanol and sonicated for 30 min. Samples were incubated at 37 ^oC for 2 h and evaporated under a stream of nitrogen. Immediately prior to the analysis, samples were resuspended in methanol, sonicated and briefly centrifuged. The LC-MS/MS analyses of sphingolipids were performed using Agilent 6460 triple quadrupole mass spectrometer coupled with the Agilent 1200 Rapid Resolution HPLC (Agilent Technologies, Santa Clara, CA) in positive mode by multiple reaction monitoring technique. The HPLC mobile phases consisted of methanol-H_2O-formic acid (74:25:1, v/v/v; RA) and methanol-formic acid (99:1, v/v; RB); both RA and RB contain 5 mM ammonium formate [161]. For measurement of ceramide and sphingoid bases, Agilent column XDB-C18, particle size 1.8 μm, 4.6 x 50 mm was used with isocratic run (100% RB, for ceramide) or gradient (0-1min, 20% B, 10-13min, 100%B and 15-20min at 20%B for sphingoid bases). The source parameters were as follows: gas temperature, 350^oC; gas flow rate, 10 L/min; nebulizer pressure, 50 psi; capillary voltage, 3500 V; The fragmentor voltage was 100 V and collision energy was 20 V.

3.3.7 Statistical Analysis

The students t-test was used to determine statistical differences between treatment and control values. Differences were considered statistical significant at $p < 0.05$.

3.4 Results

3.4.1 pJNK2/1 and CHOP is upregulated in γT induced apoptosis

Since previous data showed that γT induces apoptosis via upregulation of DR5 protein expression [27] and γT3 induced DR5 dependent apoptosis via ERS mediated JNK/CHOP [32], it was of interest to see if events similar to those reported for γT3 were activated in γT induced apoptosis. Hence, we analyzed the impact of γT on the induction of pJNK and ERS inducible protein CHOP that plays a critical role in apoptosis. Treatment of MCF-7, MDA-MB-435 and SUM 159 breast cancer cells with increasing doses of γT for 3 days induced increased levels of pJNK 2/1, CHOP and DR5 long/short (L/S) in a dose response manner (Figure 3.1A). Similar results were observed in MCF-7/TamR and MCF-7/ADR cell lines (Figure 3.1B).

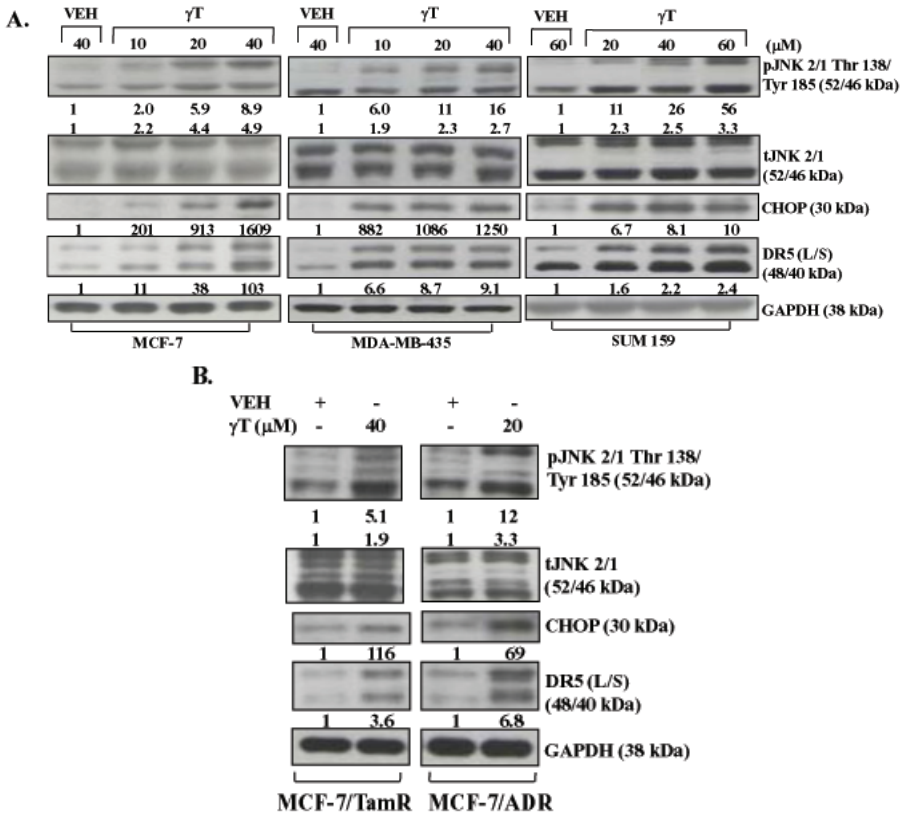


Figure 3.1: pJNK and CHOP is upregulated in γT induced apoptosis.Cells were treated with increasing doses of γT for 3 days followed by western blot analyses to determine protein levels (A and B). Data in A and B are representative of two or more independent experiments.

3.4.2 γT induces endoplasmic reticulum stress (ERS)and decreased anti-apoptotic mediators c-FLIP, Bcl-2 and Survivin protein levels

Proof that γT was inducing ERS was generated by assessing protein expression levels of known ERS markers. Treatment of cells with γT for 3 days induced increased levels of ERS markers GRP78, ATF4 and peIF2α in a dose dependent manner (Figure 3.3A), suggesting that γT induces ERS.Treatment of cells with increasing doses of γT for 3 days also decreased anti-apoptotic mediators c-FLIP (L), Bcl-2 and Survivin protein levels (Figure 3.3 B). These

data show that γT induced apoptosis is accompanied by suppression of anti-apoptotic mediators.

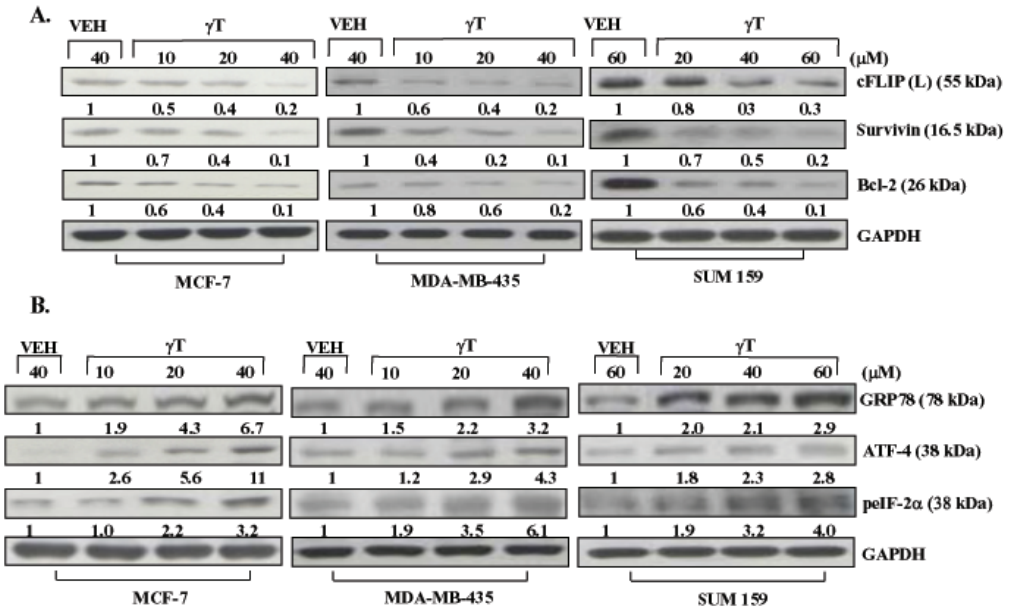


Figure 3.2: γT induced ERS and reduced anti-apoptotic factors c-FLIP, Bcl-2 and Survivin.The protein levels of ERS-related factors GRP78, ATF-4 and peIF-2α as well as anti-apoptotic factors c-Flip, Bcl-2 and Survivin were determined by western blot analyses. Data are representative of two or more independent experiments.

3.4.3 γT upregulation of pro-apoptotic and anti-apoptotic factors as well as apoptosis is JNK dependent

Since γT3 induced apoptosis was shown to be mediated by JNK dependent activation of CHOP and DR5 [32], it was important to determine if γT induced apoptosis involved a similar pro-apoptotic axis. siRNA to JNK, CHOP and DR5 significantly reduced γT induced apoptosis as detected by annexin V (Figure 3.3A) and PARP cleavage (Figure 3.3B), and markedly

reduced pJNK/CHOP/DR5 protein levels (Figure 3.3B), showing that JNK is an upstream mediator of CHOP and DR5 in γT induced apoptosis. These siRNA knockdown outcomes also suggest a cyclic loop rather than a sequential series of events. Furthermore, siRNA to JNK, CHOP or DR5 reduced the ability of γT to downregulate anti-apoptotic mediators cFLIP (L), Bcl-2, and Survivin (Figure 3.3B). Data suggest that these anti-apoptotic mediators are downregulated by JNK/CHOP/DR5 during apoptosis triggered by γT.

3.4.4 *De novo* ceramide synthesis pathway is involved in both γT and γT3 induced apoptotic events

Previous data show that the *de novo* ceramide synthesis pathway is involved in both γT and γT3 induction of apoptosis of human prostate cancer cells [28, 160]. Here, we determined if the *de novo* ceramide synthesis pathway is involved in γT and γT3 induced apoptotic events in human breast cancer cells. Treatments of MCF-7 cells with γT and γT3 for 3 days induce apoptosis in a dose-dependent manner (Figure 3.4A). Furthermore, γT3 was observed to be more effective than γT. Pre-treatment of MCF-7 cells with either of two *de novo* ceramide synthesis inhibitors: Myriocin (My) or C8-cyclopropenylceramide (C8CPPC) for 2 hours followed by treatment with γT or γT3 for 3 days significantly reduced the ability of γT and γT3 to induce apoptosis as determined by annexin V analyses (Figure 3.4B) and cleavage of caspases-8 and -9; as well as, PARP (Figure 3.4C). Furthermore, the two inhibitors reduced the ability of γT and γT3 to up-regulate pJNK, CHOP and DR5 (L/S) protein levels, and to downregulate c-FLIP (L), Bcl-2 and Survivin

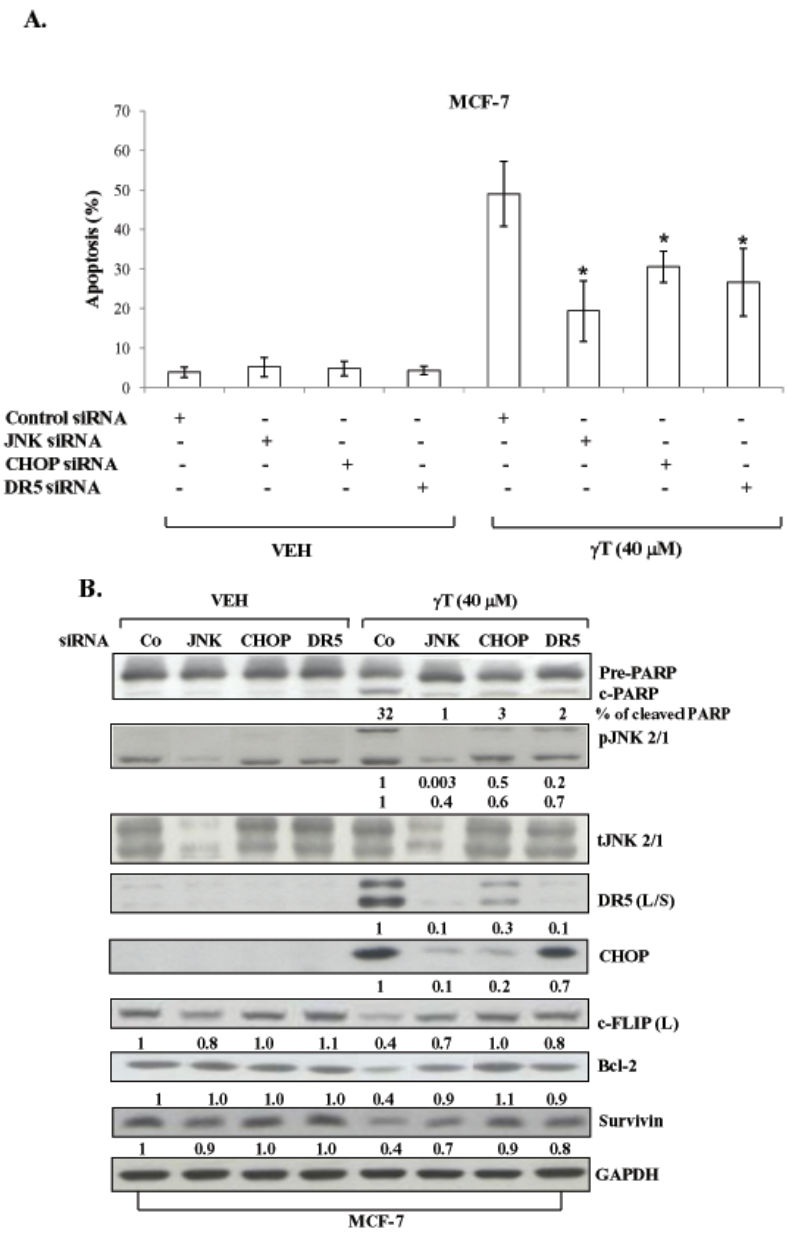


Figure 3.3: γT induced apoptosis, upregulation of pro-apoptotic mediators as well as downregulation of anti-apoptotic mediators are JNK, CHOP and DR5 dependent. Cells transfected with siRNA to JNK2, CHOP, DR5 or control were treated with γT for 3 days followed by Annexin V/FACS analyses and western blot analyses to determine apoptosis (A) and protein levels (B), respectively. Data in B are representative of two or more independent experiments. Data in A are depicted as the mean $\pm$ S.D. of three independent experiments. *significantly reduced in comparison to control siRNA, $P < 0.05$.

(Figure 3.4C). These data suggest that apoptotic events induced by γT and γT3 involve *de novo* ceramide synthesis pathway.

3.4.5 γT and γT3 induce increased levels of cellular ceramides and dihydroceramides

To further investigate the involvement of ceramides in γT- and γT3-induced apoptosis, we determined the effects of γT and γT3 on intracellular ceramide levels. Results show that treatment with either γT or γT3 for 2 or 3 days induced increased levels of 16-ceramide (16 Cer), 24-ceramide 24:1 [24 Cer (24:1)] and total ceramide (total Cer) (Figure 3.4D) and increased the levels of 16-dihydroceramide (DH-16 Cer), 24-dihydroceramide 24:1 [DH-24 Cer (24:1)] and total dihydroceramide (DH-total Cer) (Figure 3.4E) compared with VEH control. γT or γT3 treatment also induces increased levels of C18-, C20-, C22-, C24-(24:0) and C26-ceramide; as well as, dihydroceramide and dihydrosphingosine (data not shown). Taken together, these data further support a role for *de novo* ceramide synthesis in γT- and γT3-induced apoptosis.

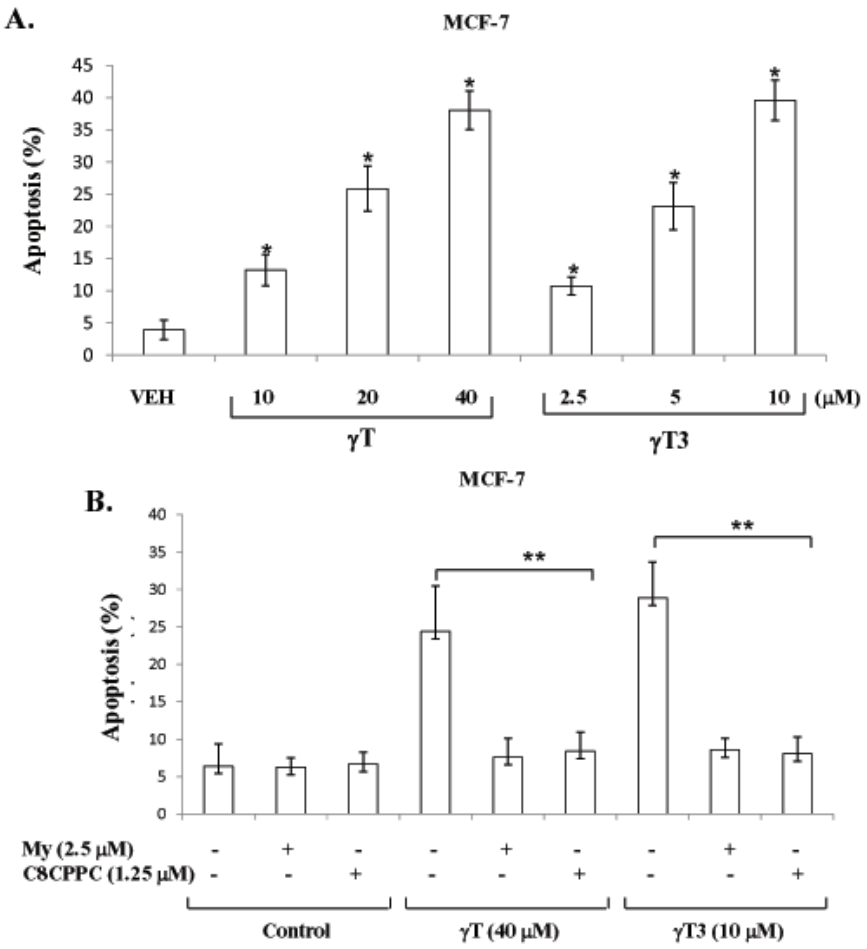


Figure 3.4: A and B

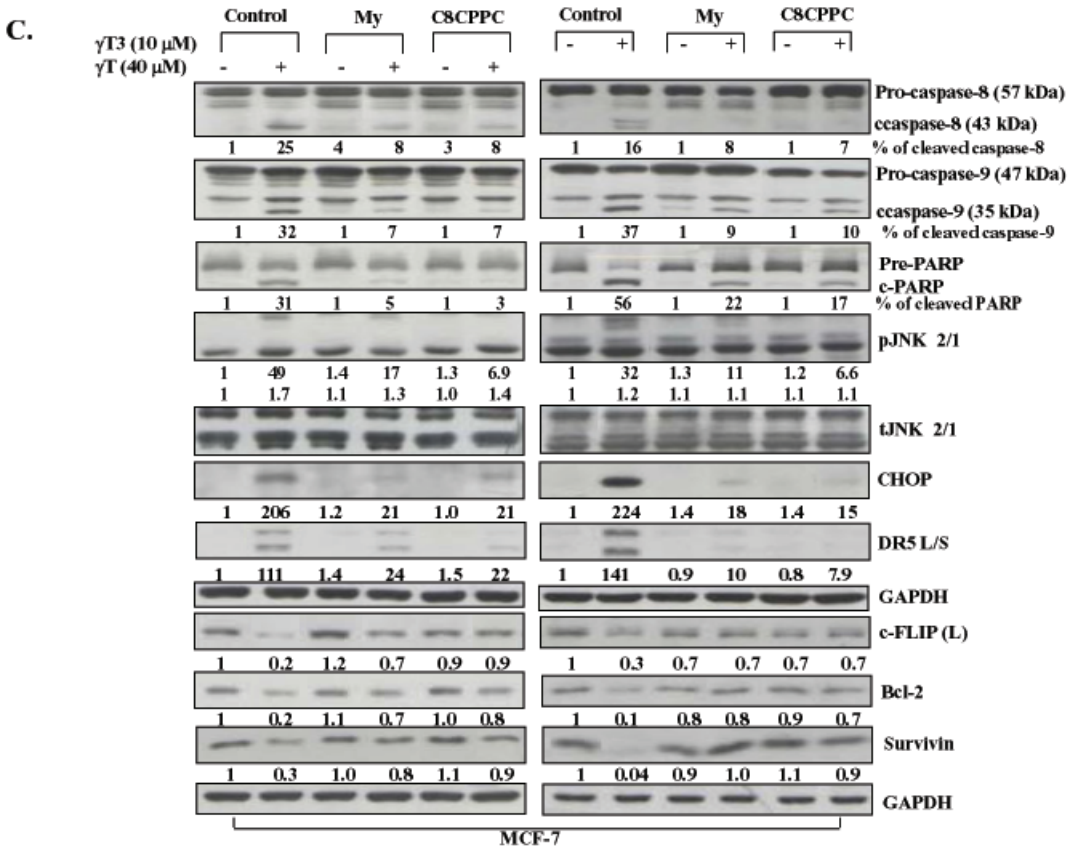

C.
Control
My
C8CPPC
Control
My
C8CPPC
γT3 (10 μM)
γT (40 μM)
- + - + - +
- + - + - +
Pro-caspase-8 (57 kDa)
ccaspase-8 (43 kDa)
1 25 4 8 3 8
1 16 1 8 1 7
% of cleaved caspase-8
Pro-caspase-9 (47 kDa)
ccaspase-9 (35 kDa)
1 32 1 7 1 7
1 37 1 9 1 10
% of cleaved caspase-9
Pre-PARP
c-PARP
1 31 1 5 1 3
1 56 1 22 1 17
% of cleaved PARP
pJNK 2/1
1 49 1.4 17 1.3 6.9
1 32 1.3 11 1.2 6.6
1 1.7 1.1 1.3 1.0 1.4
1 1.2 1.1 1.1 1.1 1.1
tJNK 2/1
CHOP
1 206 1.2 21 1.0 21
1 224 1.4 18 1.4 15
DR5 L/S
1 111 1.4 24 1.5 22
1 141 0.9 10 0.8 7.9
GAPDH
c-FLIP (L)
1 0.2 1.2 0.7 0.9 0.9
1 0.3 0.7 0.7 0.7 0.7
Bcl-2
1 0.2 1.1 0.7 1.0 0.8
1 0.1 0.8 0.8 0.9 0.7
Survivin
1 0.3 1.0 0.8 1.1 0.9
1 0.04 0.9 1.0 1.1 0.9
GAPDH
MCF-7

Figure 3.4: C

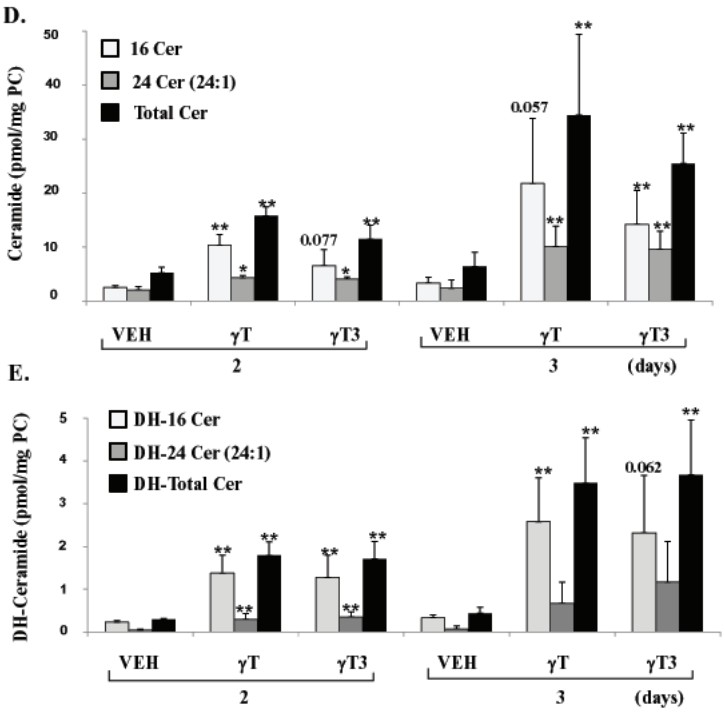


Figure 3.4: De novo ceramide synthesis pathway is involved in γT- and γT3-mediated apoptotic events.Cells were treated with increasing doses of γT and γT3 for 3 days followed by Annexin V/FACS analyses to determine apoptosis (A). Cells were pre-treated separately with two de novo ceramide synthesis inhibitors, Myriocin (My) or C8-Cyclopropenylceramide (C8CPPC) for 2 hours, followed by γT and γT3 for 3 days. Apoptosis was evaluated by Annexin V/FACS analyses (B) and protein levels were determined by western blot analyses (C). The levels of cellular ceramides (D) and dihydroceramides (E) were determined using methods described in the Material and Methods section. Data in A, B, D and E are depicted as the mean $\pm$ S.D. of three independent experiments. Data in C are representative of two or more independent experiments. *significantly increased in comparison to VEH control, $P < 0.05$. **significantly reduced in comparison to γT or γT3 treatment alone, $P < 0.05$.

3.4.6 Sphingomyelin hydrolysis pathway is involved in γT and γT3 induced apoptotic events

Treatment of MCF-7 breast cancer cells with 2.5 μM ASM inhibitor, Despiramine (Des), an inhibitor of acid spingomyelinase for 2 hours, followed by treatment with 40 μM γT or 10 μM γT3 for 3 days reduced the ability of γT or γT3 to induce MCF-7 cells to undergo apoptosis as determined by Annexin V and cleavage of PARP (Fig 3.5 A & B), activation of caspases 8 and 9 (Fig 3.5 B), increased levels of pJNK2/1, CHOP and DR5 (L/S), and decreased anti-apoptotic mediators c-FLIP, Bcl-2, and Survivin (Fig 3.5 C). These data show that ceramide synthesis is critical to the apoptotic inducing properties of γT and γT3, and that inhibition of ceramide generation via hydrolysis by ASM inhibits the ability of γT or γT3 to induce apoptotic cell death.

Based on data presented and published [27, 28, 159, 160, 162], we propose the following signaling events in γT and γT3 induced apoptosis of human breast cancer cells (Figure 3.6). Treatment of human breast cancer cells with γT or γT3 activates both the *de novo* ceramide synthesis pathway and generation of ceramide by ASM hydrolysis of sphingomyelin, resulting in enhanced levels of ceramide in cell surface membranes, and induction of endoplasmic reticulum stress. Endoplasmic stress mediators JNK/CHOP/DR5 inhibit anti-apoptotic mediators c-FLIP, Bcl-2, and survivin, and activate DR5 creating a DR5/JNK/CHOP/DR5 amplification loop involving caspase 8 and 9 mediated apoptosis.

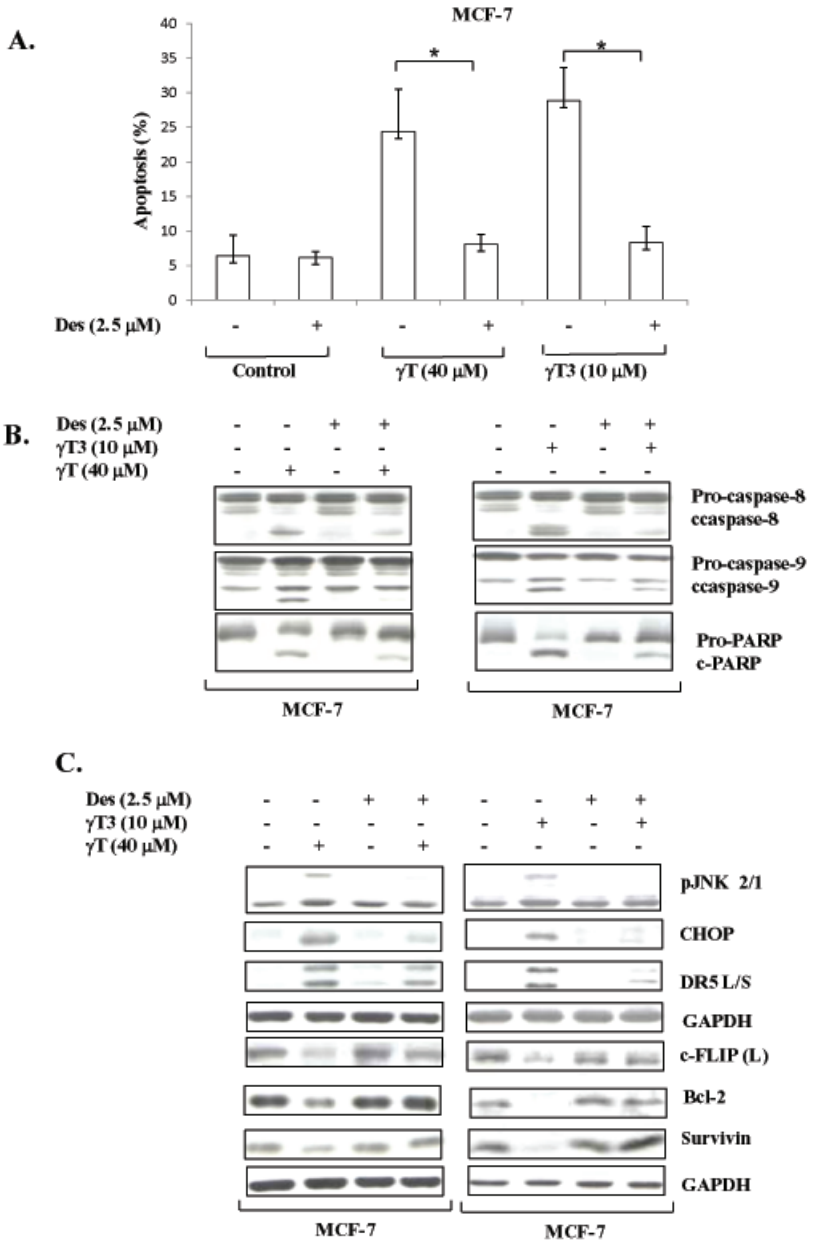


Figure 3.5: Sphingomyelin hydrolysis pathway is involved in γT- and γT3-mediated apoptotic events. MCF-7 cells were pre-treated with Despiramine (Des), an inhibitor of acid spingomyelinase for 2 hours, followed by treatment with 40 μM γT or 10 μM γT3 for 3days. Apoptosis was evaluated by Annexin V/FACS analyses (A). Cleaved PARP (c-PARP), caspase-8, caspase-9, p-JNK (2/1), CHOP, DR5, GRP78, c-FLIP (L), Bcl-2 and Survivin protein levels were determined by western blot analyses (B and C). B and C data are representative of two or more independent experiments. A data are presented as the mean S.D. of three independent experiments. *significantly reduced in comparison to γT and γT3 treatments, $P < 0.05$.

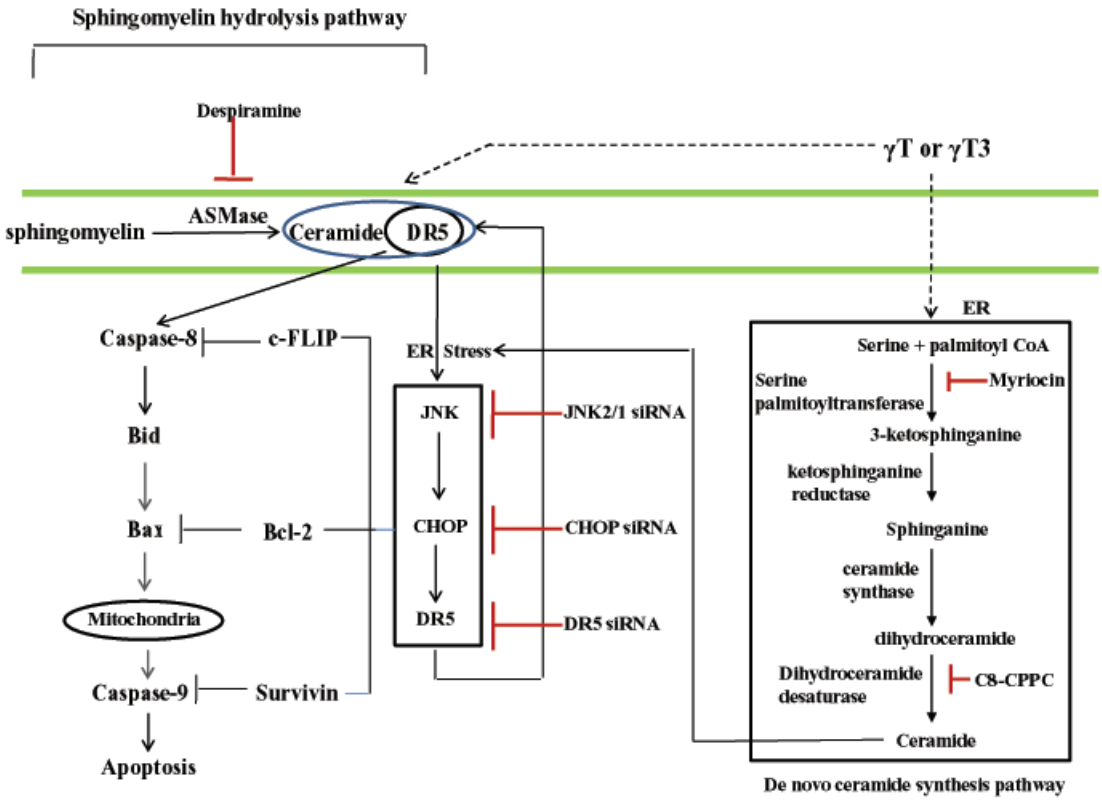


Figure 3.6: Proposed signaling events for γT and γT3 induced apoptosis in human breast cancer cells.Based on published data and data present we proposed the following signaling events that may be involved in γT and γT3 induced apoptosis of human breast cancer cells; (i) γT or γT3 induce elevated levels of cellular ceramide via de novo ceramide synthesis pathway and sphingomyelin hydrolysis pathway, leading to induction of ERS, (ii) γT or γT3 upregulates JNK/CHOP/DR5 positive loop via ERS, (iii) Upregulation of JNK/CHOP/DR5 positive loop triggers caspae-8 and caspase-9 (including mitochondria) dependent apoptotic cascade, and downregulates anti-apoptotic factors c-FLIP, Bcl-2 and Survivin, and (iv) Downregulation of the anti-apoptotic factors promotes γT- or γT3-induced apoptosis via releasing the blockage of caspase-8, Bax and capase-9, respectively.

3.5 Discussion

Although γT and γT3 possess anticancer actions, a complete understanding of the complex mechanisms involved remains unclear. Here, for the first time, studies demonstrate that γT, in a similar manner to γT3, induces apoptosis in human breast cancer cells via activation of JNK/CHOP/DR5 mediated pro-apoptotic events and suppresses anti-apoptotic factors, c-FLIP, Bcl-2 and Survivin, which are dependent; at least in part, on elevated levels of ceramide generated by the *de novo* ceramide synthesis pathway. Novel findings include: (i) Like γT3, γT induces apoptosis of human breast cancer cells via upregulation of pJNK, CHOP and DR5 protein expression, (ii) Although γT has been reported previously to decrease the expression of the cellular pro-survival protein Survivin [27], these studies show that apoptosis induced by both γT and γT3 involves simultaneous downregulation of three key anti-apoptotic factors c-FLIP, Bcl-2, and Survivin, (iii) Both γT and γT3 induce increased levels of total cellular ceramides and dihydroceramides, and (iv) *De novo* ceramide synthesis and sphingomyelin hydrolysis is involved in both γT and γT3 apoptotic events. Taken together, these studies provide a better understanding of signaling pathway elements that can be successfully targeted to achieve improved clinical benefit in cancer prevention and therapy. Activation of DR5, leading to cell death, has been observed to specifically occur in cancerous cell types while sparing normal cells [163]. This has led to the development and use of DR4/DR5 targeted antibody therapies [163]. A number of anticancer agents have been identified as mediating their anticancer actions at least in part via upregulation of DR5 including

several vitamin E compounds; namely, VES [164], α-TEA [165], γT [27] and γT3 [32]. DR5 can be upregulated by multiple factors such as NF-κB, p53, JNK and CHOP [86, 87, 166, 167]. Previous data show that ERS contributes to both α-TEA- and γT3-induced DR5 dependent apoptosis via activation of JNK/CHOP [32, 137]. Here, we reported that γT induced DR5 mediated apoptosis also requires JNK and CHOP. Since data also show that γT induces ERS we hypothesized that, like γT3, γT upregulation of JNK/CHOP/DR5 is ERS dependent. c-FLIP, Bcl-2 and Survivin play important protective roles in caspase-8 and mitochondria mediated apoptotic cascades. It is not surprising that c-FLIP and Bcl-2 are downregulated by γT via JNK/CHOP/DR5 pathway, since we have reported that α-TEA downregulated c-FLIP and Bcl-2 via the same pathway [137]. However, the finding that Survivin downregulation in γT mediated apoptosis is also regulated by JNK/CHOP/DR5 pathway is totally unexpected. How JNK/CHOP/DR5 pathway downregulates Survivin is not known. CyclinD1 has been reported to target Survivin by degradation in a G1 block dependent manner [143] and cyclin D1 in turn has been shown to be down-regulated at protein levels by ERS [168]. Thus, it is possible that ERS activation contributes to Survivin degradation in γT mediated apoptosis. This hypothesis is supported by data showing that (i) both γT and γT3 induce ERS (Figure 4) [32] and G1 cell cycle arrest [12]; as well as, down-regulate cyclinD1 (data not shown) and (ii) γT downregulation of Survivin occurs at the protein level, but not at the mRNA level (data not shown). In agreement with previous reports in prostate cancer cells [28, 160], data presented here show that the *de novo* ceramide synthesis pathway is involved in

γT- and γT3-induced apoptosis in breast cancer cells. Importantly, our data showing that inhibition of *de novo* ceramide synthesis using inhibitors; Myriocin and C8CPPC that specifically inhibit both the first and final enzymes in the *de novo* pathway, respectively, blocked γT and γT3 induced apoptotic events suggest that ceramide generated by the *de novo* synthesis pathway is involved in γT and γT3 induced apoptosis in human breast cancer cells. Data showing that γT and γT3 induce increased levels of total ceramide, further support involvement of ceramide generation in γT and γT3 induced apoptosis. Also, data documenting increases in the levels of dihydroceramides, an important intermediate in the *de novo* pathway, adds further support for the involvement of the *de novo* ceramide pathway in γT and γT3 mediated effects. It is interesting to note that unlike previous studies showing that γT and γT3 induce accumulation of dihydroceramide and dihydrosphingosine without affecting ceramide during the early-stage of γT or γT3 treatment in prostate cancer cells [28, 160], here we observe that these vitamin E forms increase both cellular ceramide and dihydroceramide in breast cancer cells. These cell type dependent effects may be due to different activities of key enzymes involved in ceramide metabolism, which warrants further investigation. Sphingomyelin hydrolysis has been shown to be activated in response to stress stimuli generated by death receptors, chemotherapeutic agents, infections, gamma and UV radiation [154]. Previously, sphingomyelin hydrolysis was shown to be involved in apoptosis induced by a modified form of vitamin E, γ-TEA [158]. Interestingly, data reported here show that sphingomyelin hydrolysis is also involved in apoptotic events induced by γT and γT3. Data showing that both

the *de novo* ceramide synthesis pathway and sphingomyelin hydrolysis simultaneously contribute to γT and γT3 induced ER-stress and apoptosis are in agreement with the work done by Sauane et al 2010 in prostate cancer cells treated with novel therapeutic agent recombinant melanoma associated gene-7/interleukin 24 (mda7/IL24). However, the mechanisms whereby γT and γT3 regulate both *de novo* ceramide synthesis and sphingomyelin hydrolysis are not understood. The regulation of ceramide synthesis pathways is complicated. It is not clear if both agents individually activate distinct upstream mediators or common mediators to induce both sphingomyelin hydrolysis and *de novo* synthesis simultaneously or the two pathways are activated in a specific sequence. Further work will have to be carried out to identify the sequence of events. Based on published data and data present here a schematic diagram of proposed signaling events in γT and γT3 induced apoptosis of human breast cancer cells are depicted in Fig 3.6. In conclusion, these studies show that the naturally occurring forms of vitamin E, γT and γT3, induce apoptosis through activation of pro-apoptotic JNK/CHOP/DR5 mediated events and down-regulation of anti-apoptotic factors c-FLIP, Bcl-2 and Survivin via increased levels of ceramide generated by the *de novo* ceramide synthesis and sphingomyelin hydrolysis pathway. These studies provide insights for better understanding of the anticancer actions of vitamin E and increased knowledge into how targeting ERS can mediate pro-apoptotic pathways which may be beneficial in prevention and therapy of human breast cancer.

Chapter 4

Eliminating tumor initiating and bulk drug resistant breast cancer cells with combination of simvastatin and gamma-tocotrienol

4.1 Abstract

This study examines the anticancer action of simvastatin (SVA) and gamma-tocotrienol (γT3) alone and in combination on eliminating tumor initiating cells (TICs) and non-TICs in drug resistant human breast cancer cell lines; namely, acquired tamoxifen (Tam) resistant MCF-7 (MCF-7/TamR) and acquired adriamycin/doxorubicin (AD) resistant MCF-7 cells (MCF-7/ADR). Here, we report that both drug resistant cell lines contain higher percentages of aldehyde dehydrogenase activity positive (ALDH+) and CD44+/CD24- populations in comparison with the parental cell lines. ALDH+ populations from both cell lines exhibit the capacity to self-renew and ability to form colonies, two characteristics of TICs, as determined by mammosphere and colony formation assays, respectively, indicating that TICs are enriched in ALDH+ populations. Both drug resistant cell lines expressed higher levels of signal transducer and activator of transcription 3 (Stat-3) phosphorylated at tyrosine-705 (pStat-3, Tyr-705), c-Myc, CyclinD1, Bcl-xL and Survivin in comparison with their parental cell lines and pStat-3, c-Myc, Cyclin D1, Bcl-

xL and Survivin are highly expressed in ALDH+ in comparison with either ALDH- or unsorted cells. Knockdown of Stat-3 using a chemical inhibitor of Stat-3, reduced ALDH+ and CD44+/CD24- populations, inhibited mammosphere formation, and decreased pStat-3, c-Myc, CyclinD1, Bcl-xL and Survivin protein expression, indicating that Stat-3 is critical for maintaining TICs in these cell lines. SVA and γT3 alone and in combination reduced ALDH+ and CD44+/CD24- populations, inhibited mammosphere formation, and decreased pStat-3, c-Myc, CyclinD1, Bcl-xL and Survivin protein expression in both unsorted and ALDH+ population. Furthermore, SVA and γT3 either singly or in combination induced apoptosis which is associated with activation of JNK/CHOP/DR5 pro-apoptotic events that have been reported previously to be involved in both SVA and γT3 induced apoptosis in parental MCF-7 cells. Taken together, these *in vitro* data demonstrate that drug resistant human breast cancer cell lines contain higher levels of a TIC enriched population which is regulated in part via highly expressed pStat-3, and that the combination of SVA plus γT3 has the ability to target both the enriched TIC and bulk cancer cell populations .

4.2 Introduction

Breast cancer is the second leading cause of cancer death in women in the U.S [1]. Despite great advances in therapeutic strategies devised specifically to target various sub-types of breast cancer, acquisition of drug resistance and treatment associated side effects compromise effectiveness. Hence, a better understanding of the molecular changes observed in drug resistance and

strategies to overcome it with low/nontoxic interventions is required to improve the overall disease-free survival rates. Accumulating data support the concept that breast tumors originate from breast cancer stem and/or progenitor cells, which are referred to as either tumor initiating cells (TICs) or cancer stem cells (CSCs) [169]. TICs represent a small population of cancer cells that exhibit self-renewal and differentiation characteristics similar to normal stem cells but differ in that their self-renewal pathways are deregulated [107, 169]. Based on the TIC concept, TICs are responsible for tumor formation, progression, metastasis, and recurrence; as well as, drug resistance [107, 170]. The concept of TICs has profound implications for early detection, prevention, and treatment of cancer [107, 170, 171]. Thus, targeting TICs provides a promising approach for cancer prevention and treatment, especially for drug resistant breast cancer.

Signal transducer and activator of transcription 3 (Stat-3) is activated in response to cytokine and growth factor signaling. Activation of stat-3 by Janus Associated Kinase (JAK) or growth factor receptor-associated tyrosine kinase (Src) involves phosphorylation of 705 tyrosine residue at C-terminal domain. Phosphorylated Stat-3 forms homo- or hetero-dimer that translocate to the nucleus and binds to DNA binding domains of the regulated genes [121, 172]. Active Stat-3 has been observed to be highly expressed in multiple tumors including breast cancer. Activated Stat-3 can transcriptionally regulate pro-proliferation or TIC-factors such as c-Myc, CyclinD1, Bcl-xL and Survivin in addition to regulating genes associated with invasion and angiogenesis. Although breast cancer cell lines and tumor tissues have been identified

to express high levels of active Stat-3, the role of Stat-3 in development of drug resistance and associated TIC in breast cancer has not been identified [121, 172].

Presently, treatments including anti-hormones such as tamoxifen (Tam), chemotherapeutic such as adriamycin/doxorubicin (ADR/DOX), and biological agents, are used in both adjuvant and neo-adjuvant settings. They have been observed to be effective in 90% of primary breast cancers and 50% of metastases at the start of the therapeutic period. However, eventually resistance to therapy and toxic side effects develops [2, 173, 174]. At this stage the tumors may also present with cross-resistance to other therapeutic agents. A number of molecular changes have been identified to be associated with progression of therapeutic resistance and recurrent disease [2, 173, 175], including increased cross-talk between various receptor tyrosine kinase signaling pathways, alterations of apoptotic mediators and DNA repair mechanisms, decreased activation of drug; as well as, increased drug efflux from the cells [2, 173, 175]. However, the complete accounting of mechanisms involved has not been achieved. Simvastatin (SVA) is one of the family of statin drugs that are the most widely prescribed drugs used for the reduction of cholesterol levels via inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step in mevalonate synthesis [130]. A growing body of information over the past decade suggests that statins possess potential for use in cancer management [50–56, 130, 131]. Numerous *in vitro* and preclinical studies have shown that various statins, particularly lipophilic statins such as SVA have antiproliferative, antiangiogenic,

antimetastasis and pro-apoptotic properties in many types of cancer cells including breast cancer [50–56, 130, 131]. Moreover, Gauthaman et al., provided evidence for anti-TIC effects of SVA in karyotypically abnormal mouse embryonic stem cells [176]. Vitamin E consists of a group of structurally distinct naturally occurring compounds that are classified as tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ). The general term vitamin E is also used for synthetic vitamin E (all-rac-α-tocopherol); as well as, vitamin E analogs such as vitamin E succinate (VES) and RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA). Some, but not all vitamin E compounds, display anticancer properties *in vitro* and *in vivo* [24]. Of the naturally occurring forms, accumulating evidence supports that γT3 possesses potent anticancer properties, including inhibition of tumor growth *in vivo* and inhibition of cell proliferation, metastasis and angiogenesis; as well as, induction of apoptosis *in vitro*. Recently, γT3 has been reported to have the ability to eliminate TICs in prostate cancer [124]. The combination of SVA plus γT3 has been shown to exhibit better anticancer actions than either agent by itself in mammary cancer [177]. Here, we investigated the anticancer effects of SVA and γT3 singly; as well as, in combination in targeting both TIC and bulk tumor cells from drug resistant human breast cancer cell lines; namely, MCF-7/TamR and MCF-7/ADR. We report that both SVA and γT3 alone and in combination target TIC via elimination of TIC enriched population and bulk tumor cells via induction of apoptosis, suggesting that the combination of SVA + γT3 is a potential regimen for prevention and treatment of drug resistant breast cancer.

4.3 Materials and Methods

4.3.1 Chemicals

Simvastatin sodium salt (SVA), an active form of simvastatin *in vitro*, was obtained from Calbiochem (San Diego, CA). JSI-124 (Cucurbitacin I), an inhibitor of phosphorylation of Stat3 at Tyr-705 [178], was purchased from Calbiochem (Cambridge, MA). γT3 was a gift from Malaysian Palm Oil Board (Kuala Lumpur, Malaysia).

4.3.2 Cell Culture

MCF-7/ADR and MCF-7/TamR cells which were derived by selection of parental cells in the presence of doxorubicin and tamoxifen respectively, were obtained from Dr. Kapil Mehta, (M.D Anderson Cancer Center, Houston TX) and Dr. Linda deGraffenried (University of Texas at Austin, Austin, TX), respectively. Cells were maintained and cultured as published before [133, 179]. For experiments, FBS was reduced to 2%. γT3 was dissolved in 1:4 (vol/vol) DMSO/ethanol at 40 mM as stock solution. SVA was dissolved in ethanol. Equivalent levels of 1:4 (vol/vol) DMSO/ethanol was used as vehicle control (VEH).

4.3.3 Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturers instructions (Invitrogen, Carlsbad, CA) and previously published procedures from our lab [32]. Annexin V/FITC was a gift from Dr. Shawn Bratton, (M. D. Anderson Cancer Center, Smithville, TX).

4.3.4 Western blot analyses

Western blot analyses were conducted as described previously [32]. Primary antibodies to the following proteins were used in this study; PARP, GRP-78, pJNK, CHOP, c-Myc, CyclinD1, Bcl-xL and Survivin (Santa Cruz Biotechnology, Santa Cruz, CA), DR5, Caspase-8, Caspase-9, pStat-3 and Stat-3 (Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, made in house). Secondary antibodies used included: horseradish peroxidase conjugated goat-anti-rabbit and rabbit-anti-mouse (Jackson Immunoresearch, Rockford, IL); and bovine-anti-goat (Santa Cruz Biotechnology).

4.3.5 Analysis of aldehyde dehydrogenase activity

ALDH activity was determined using an Aldefluor assay kit (StemCell Technologies, Inc, Vancouver, BC, Canada) as per the manufacturers instructions. Aldefluor fluorescence was detected using a FL1 detector in a LSRII flow cytometer and analyzed using BD FACSDiVa software.

4.3.6 Mammosphere formation and limiting dilution assays

ALDH+, ALDH- and unsorted MCF-7/TamR and MCF-7/ADR cells were assessed for ability to form mammospheres using established mammosphere formation conditions [132]. Cells were cultured in DMEM/F12 phenol red free (MCF-7/TamR) and phenol red (MCF-7/ADR) medium supplemented with fibroblast growth factor (10 ng/mL) and epidermal growth factor (10 ng/mL; Preprotech, Rocky Hill, NJ); insulin (50 μg/mL; Sigma Chemical Co); and B27 (100 units/mL), N2 supplements (100 units/mL), penicillin

(100 units/mL), and streptomycin (100 g/mL) (Invitrogen). Serial dilutions of cells were plated in 24 well ultra low attachment plates and cultured for 7 days [132]. Mammospheres were characterized as spherical organoids of more than 50 cells. The number of mammospheres was scored using microscope with regular light and 100 x magnification.

4.3.7 Analysis of the cell surface expression of CD44 and CD24 by immunostaining and flow cytometry

Cells were washed with 0.5% BSA in PBS and incubated with IgG2b species specific antibody for 15 minutes at 4^oC. CD44 and CD24 (BD Pharmingen, San Diego, CA) fluorochrome-conjugated antibodies were added as per the manufacturers instructions and incubated for 30 minutes at 4^oC. Cells were washed with 0.5%BSA in PBS and 7-AAD was added to samples prior to analysis to exclude non-viable cells. CD44-APC was detected using FL5 and CD24-PE was detected using FL2 filters in a LSRII flow cytometer and analyzed using BD FACSDiVa software.

4.3.8 Colony formation assay

400, 200 and 100 cells/12 wells were cultured on 12 well tissue culture plastic plates for 14 days followed by fixing the cells with methanol and staining with 2% (w/v) crystal violet in H_2O. Colonies containing more than 50 cells were scored [116, 180, 181].

4.3.9 Statistical Analysis

The data were analyzed using two-tailed student *t-test* for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p < 0.05$.

4.4 Results

4.4.1 Drug resistant MCF-7/TamR and MCF-7/ADR cell lines contain higher levels of ALDH+ and CD44+/CD24- TIC cell populations in comparison to the drug sensitive (MCF-7/TamS or MCF-7) parental cell lines.

Cell sorting analyses based on ALDH+ and CD44+/CD24- TIC markers show that MCF-7/TamR and MCF-7/ADR cell lines have 6.2 1.11% and 6.8 0.56% ALDH+ cells versus 0.3 0.02% and 0.1 0.05% in their MCF-7/TamS and MCF-7 parental counterparts (Fig 4.1A), and the MCF-7/TamR and MCF-7/ADR cells have 60.4 2.71% and 30.4 1.53% CD44+/CD24- cells versus 1.3 0.56% and 0.8 0.26% in their parental cell counterparts (Fig 4.1B). Furthermore, MCF-7/TamR and MCF-7/ADR cells have 5.5 0.21% and 1.25 0.07% ALDH+/CD44+/CD24- cells versus 0.2 0.1% and 0% in MCF-7/TamS and MCF-7 cells respectively (Fig 4.1C).

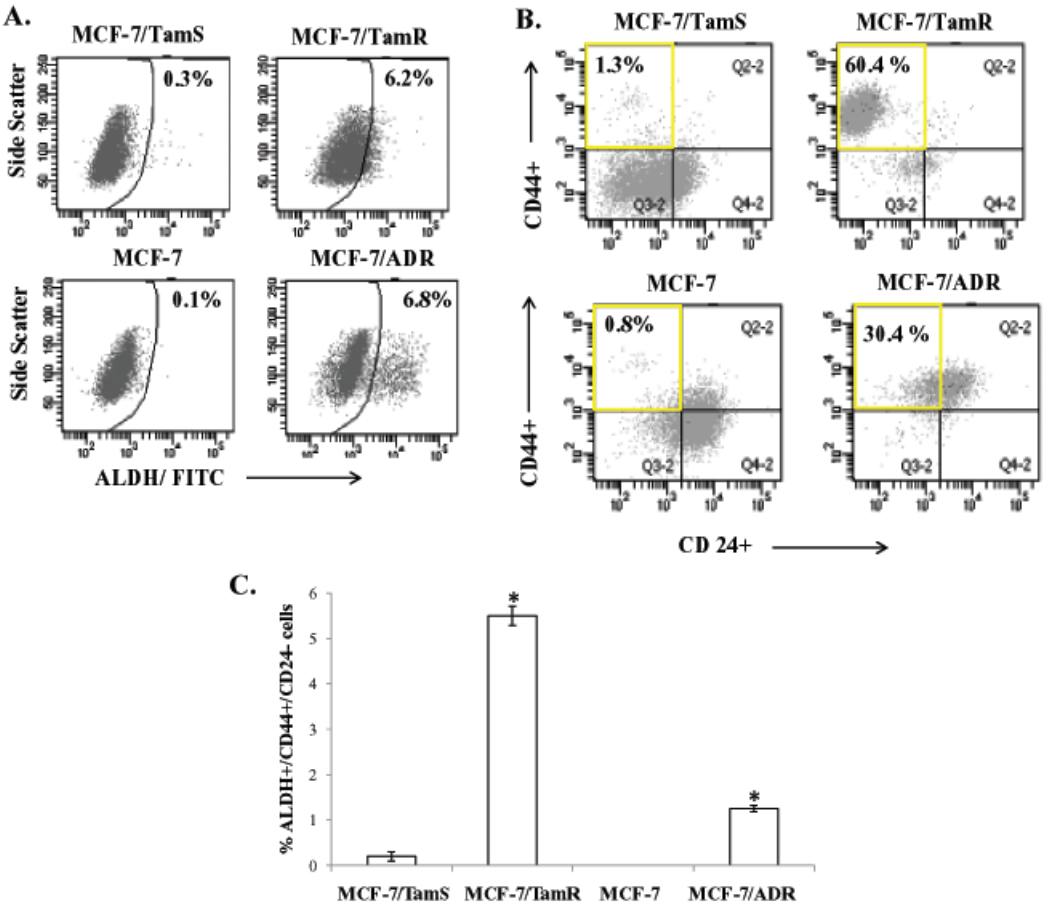


Figure 4.1: The percentages of ALDH+, CD44+/CD24- and ALDH+/CD44+/CD24- were determined using ALDH activity assay (A), CD44/CD24 immunostaining assay (B) and both assays (C), respectively. Data are representative images of three independent experiments.

4.4.2 ALDH+ populations are enriched in tumor initiating cells.

To confirm the tumor initiating properties of ALDH+ populations, MCF-7/TamR and MCF-7/ADR cells were sorted into ALDH+ and ALDH- cell populations by FACS, and examined for ability to form mammospheres in

	ALDH+ cells(%)	
	MCF-7/TamR	MCF-7/ADR
Sorted $ALDH+$ cells	6.03 ± 0.47*	6.30 ± 0.78*
Sorted $ALDH-$ cells	0.87 ± 0.38	0.07 ± 0.06

Table 4.1: Percentage of ALDH+ cells obtained from enriched ALDH+ and ALDH- cell population after 14 days of culture. ALDH positive and negative cells sorted from MCF-7/TamR and MCF-7/ADR were cultured in monolayer for 14 days. ALDH activity was determined using Aldefluor assay. *mean ± S.D. of three independent experiments.

selective media on low attachment plates, an *in vitro* characteristic of TICs [116, 132]. The ALDH+ populations from MCF-7/TamR and MCF-7/ADR cell lines, seeded at 15-2,000 and 250-4,000 ALDH+ cells, formed significantly more mammospheres in comparison with ALDH- and unsorted cells (Fig 4.2A and B). 500 seeded ALDH+ MCF-7/TamR cells and 4,000 seeded ALDH+ MCF-7/ADR cells demonstrated a 18.4 and 17.6 fold increase in their ability to form mammospheres compared to ALDH- cells seeded at the same cell concentration, respectively (Fig 4.2 A and B), indicating that TICs are enriched in ALDH+ populations in both cell lines. In the colony formation assay, the number of colonies formed by ALDH+ cells from MCF-7/TamR and MCF-7/ADR cell lines, seeded at 100, 200, and 400 cells/plate were significantly higher than the ALDH- cells treated in a like manner (Fig 4.2 C and D). The ALDH+ cells showed a 5.4 and 4.2 fold increase in colony formation compared to the ALDH- cells (Fig 4.2 C and D), respectively.

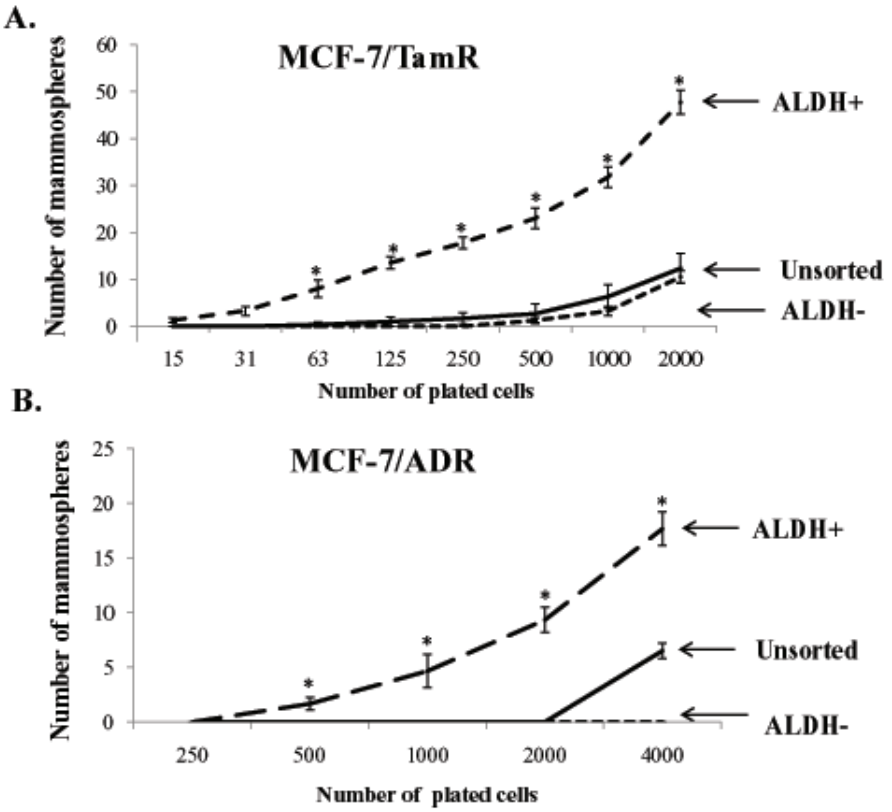
Furthermore, to determine if the FACS sorted ALDH+ cells undergo differentiation, ALDH+ enriched cells from MCF-7/TamR and MCF-7/ADR

cells were seeded in monolayer culture plates and cultured for two weeks, cells harvested and re-sorted on the basis of ALDH+. The number of ALDH+ cells for MCF-7/TamR and MCF-7/ADR cell lines were 6.03 0.47% and 6.3 0.78%, respectively (Table 4.1), approximately the same percentage of ALDH+ cells observed in unsorted cell lines (Fig 4.1 A). These data suggest, as previously reported [111, 181], that ALDH+ enriched cells grown in monolayer cultures for two weeks give rise to ALDH- cells and maintain the original ALDH+ percentage. No increase in ALDH+ cells was observed when ALDH- cells were cultured under the same conditions. Furthermore, these experiments show that ALDH- cell populations, under these conditions, do not have the capacity to revert to ALDH+ cells. These observations provide further evidence that TICs are enriched in ALDH+ populations in these drug-resistant human breast cancer cell lines.

4.4.3 Unsorted MCF-7/TamR and MCF-7/ADR breast cancer cells express high levels of phosphorylated (active) signal transducer and activator of transcription-3 (pStat-3), which is enriched in the ALDH+ sorted cell population.

To better understand the mechanisms by which TICs are maintained at the enriched levels in MCF-7/TamR and MCF-7/ADR cell lines, western blot analyses were conducted using unsorted cell extracts from the two drug resistant cell lines and their respective parental cell lines to determine protein levels of pStat-3, cyclin D1, transcription factor c-Myc, Bcl-2 family member Bcl-xL, and Survivin. All of these factors which are associated with cell division and cell survival were highly expressed in the drug resistant cells in

comparison to their parental counterparts (Fig 4.3A). Additional western blot analyses were conducted to determine levels of pStat-3 and downstream mediators in MCF-7/TamR and MCF-7/ADR ALDH+ enriched cells in comparison to ALDH- and unsorted cells. The ALDH+ population had higher protein levels of pStat-3 (Tyr-705), Cyclin D1, c-Myc, Bcl-xL and Survivin in comparison with ALDH- and unsorted populations in both drug resistant cell lines (Fig 4.3B), suggesting pStat-3 involvement in TIC proliferation and survival.



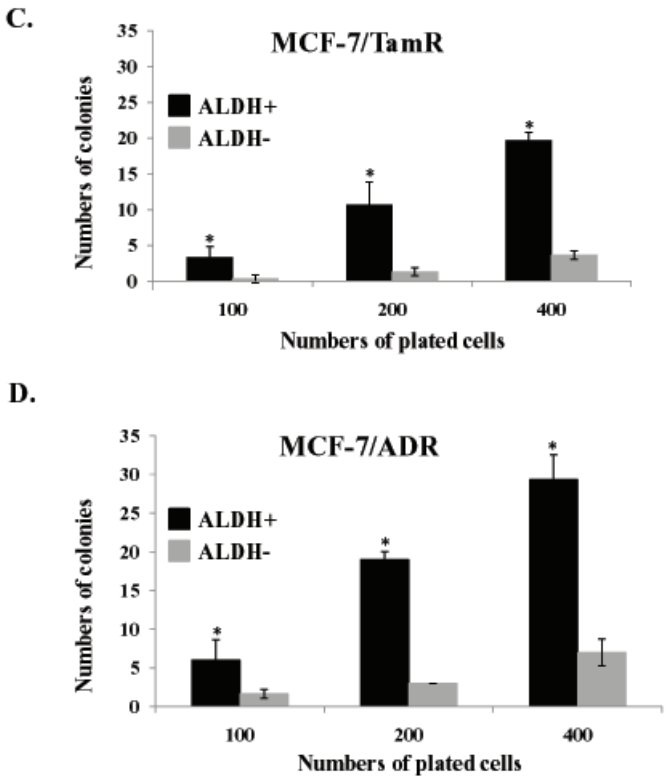


Figure 4.2: Mammosphere formation assay was performed using sorted ALDH+, ALDH- and unsorted cells (A and B). Colony formation assay was performed using ALDH+ and ALDH- cells(C and D). The numbers of mammospheres (A and B) or colonies (C and D) are presented as means ± SD of three individual experiments. *significantly different from ALDH- or unsorted cells, $p < 0.05$.

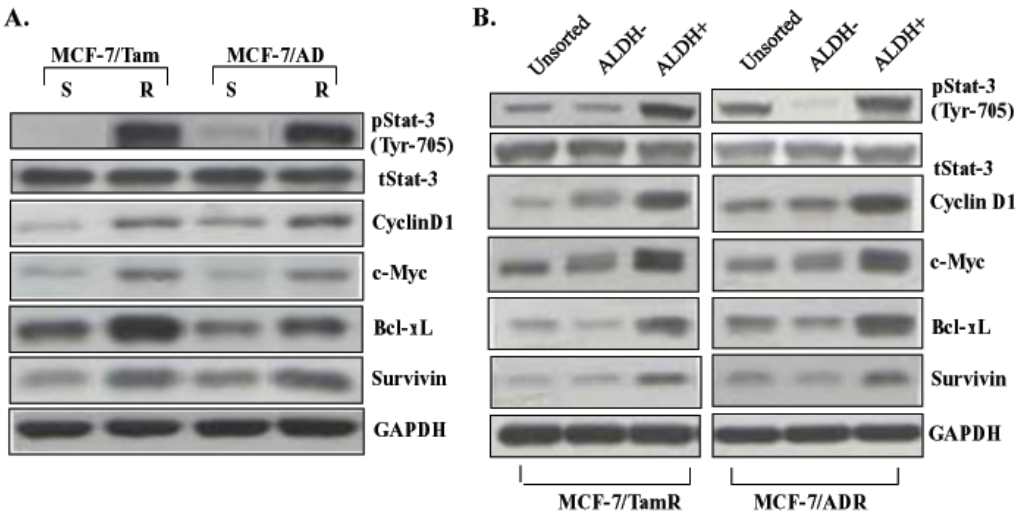


Figure 4.3: Drug resistant breast cancer cells express higher levels of pStat-3, which is enriched in ALDH+ population.Western blot analyses were performed to determine protein expression in unsorted population in comparison with their parental cell lines using GAPDH as loading control (A) and in ALDH+ population in comparison with unsorted and ALDH- populations using GAPDH as loading control (B). Data are representative of two or more independent experiments.

4.4.4 The Stat-3 signalling pathway is critical for maintaining TIC enriched populations in drug resistant breast cancer cells.

Rationale for focusing the mechanistic studies on Stat-3 comes from a study showing Stat-3 to be important for maintenance of TICs in MCF-7 breast cancer cells [182]. In order to determine if Stat-3 contributes to maintenance of TIC-enriched populations in our drug resistant cell lines, we used the mammosphere assay as a measure for number of TICs following inhibition of Stat-3 activity with the chemical inhibitor. Cucurbitacin I is a potent inhibitor of the janus kinase 2/Stat-3 signaling pathway which exhibits anti-tumor and anti-proliferative properties (Sigma Aldrich.com). Stat-3 inhibitor

	MCF-7/TamR		MCF-7/ADR	
	ALDH+	CD44+/CD24-	ALDH+	CD44+/CD24-
VEH	6.03 ± 0.30	55.75 ± 0.20	5.60 ± 0.65	28.12 ± 1.67
JSI-124 (0.125 μM)	0.37 ± 0.29*	30.35 ± 5.90*	0.45 ± 0.32*	5.01 ± 2.55*

Table 4.2: Effect of Stat3 inhibitor on TIC population. MCF-7/TamR and MCF-7/ADR were treated with vehicle control or with JSI-124 (0.125M) for 2 days followed by determination of ALDH+ and CD44+/CD24- populations using ALDH activity and CD44+/CD24- immunostaining assays, respectively. *significantly different from vehicle (VEH), $p < 0.05$ using students t-test. Mean ± S.D. of three independent experiments

significantly reduced the number of mammospheres in a dose-dependent manner in both drug resistant cell lines (Fig 4.4A). Stat-3 inhibitor also significantly reduced the ALDH+ and CD44+/CD24- populations in both drug resistant cell lines (Table 4.2).

Furthermore, western blot analyses show that Stat-3 inhibitor suppressed protein levels of pStat-3 (Tyr-705), as well as Cyclin D1, c-Myc, Bcl-xL and Survivin (Fig 4.4 B), suggesting that Cyclin D1, c-Myc, Bcl-xL and Survivin are downstream mediators of Stat3. Taken together, these data demonstrate a critical role for Stat-3 in TIC survival. Since CyclinD1, c-Myc, Bcl-xL and Survivin are established stem cell mediators [183, 184], our data suggest that high expression of active Stat-3 (pStat-3, Tyr-705) is necessary but not totally sufficient for maintenance of TIC populations in drug resistant cells.

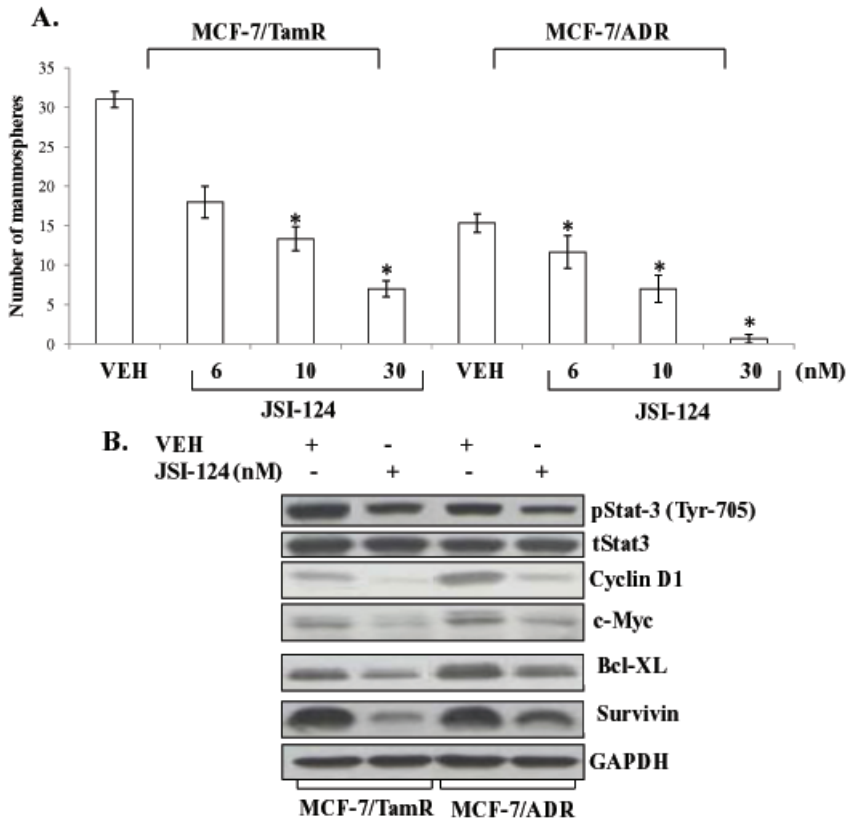


Figure 4.4: Stat-3 is critical for maintaining TIC enriched populations in drug resistant cells.ALDH+ populations were treated with indicated concentrations of STAT-3 inhibitor for 7 days to determine the number of mammospheres as detected by mammospheres formation assay (A). Western blot analyses were performed to evaluate protein expression in unsorted DR cells treated with Stat-3 inhibitor or VEH control for 2 days (B). Data are representative of two or more independent experiments. Data are presented as means SD of three individual experiments. *significantly different from VEH, $p < 0.05$.

4.4.5 Both SVA and γT3 reduce TICs and suppress pStat-3(Tyr-705), CyclinD1, c-Myc, Bcl-xL, and Survivin protein expression in both drug resistant human breast cancer cell lines.

The ability of SVA and γT3 to reduce TICs was evaluated by ALDH activity assay, staining for CD44+/CD24- and mammosphere formation of ALDH+ populations. Treatment of MCF-7/TamR and MCF-7/ADR cells with increasing levels of SVA or γT3 for 2 days significantly reduced the percentages of ALDH+, CD44+/CD24- and ALDH+/CD44+/CD24- populations in a dose-dependent manner (Fig 4.5 A, B and C). SVA at 2.5 μM or less and γT3 at 5 μM or less are pharmacologically achievable levels [138, 185], thus, these data show that SVA as well as γT3 at pharmacologically achievable levels levels eliminate TICs in drug resistant cell populations. Furthermore, treatment of ALDH+ cells obtained from MCF-7/TamR and MCF-7/ADR cells with pharmacologically achievable levels of SVA or γT3 for 1 week significantly reduced the number of mammospheres formed in a dose-dependent manner (Fig 4.5 D). These data demonstrated that SVA as well as γT3 reduces the number of TICs in drug resistant breast cancer cells. Both SVA and γT3 at pharmacologically achievable levels suppressed pStat-3(Tyr-705), CyclinD1, and c-Myc, Bcl-xL and Survivin protein levels in a dose-dependent manner in unsorted MCF-7/TamR and MCF-7/ADR cell lines (Fig 4.5 E). Furthermore, treatment of ALDH+ cells sorted from MCF-7/TamR or MCF-7/ADR cell lines with SVA at 0.625μM or γT3 at 7.5μM suppressed pStat-3 (Tyr-705) , CyclinD1, c-Myc, Bcl-xL and Survivin protein levels (Fig 4.5 F). These data suggest that downregulation of these TIC-associated mediators maybe important in SVA or γT3 mediated elimination of TICs.

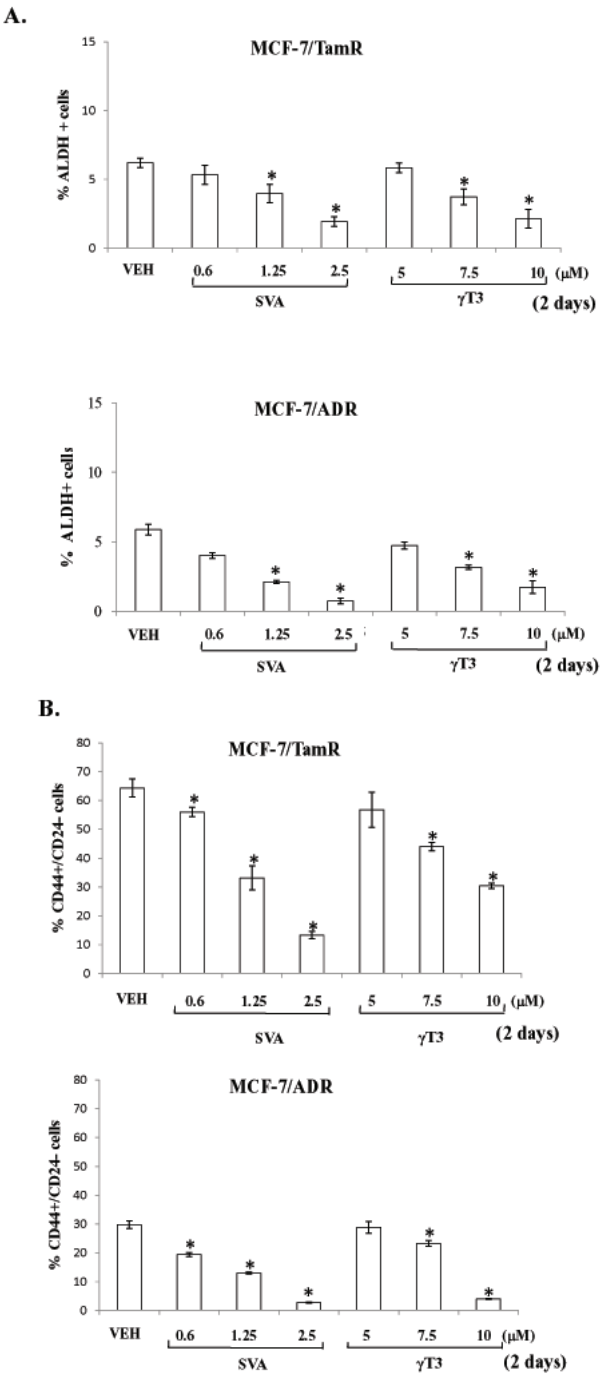


Figure 4.5: A and B

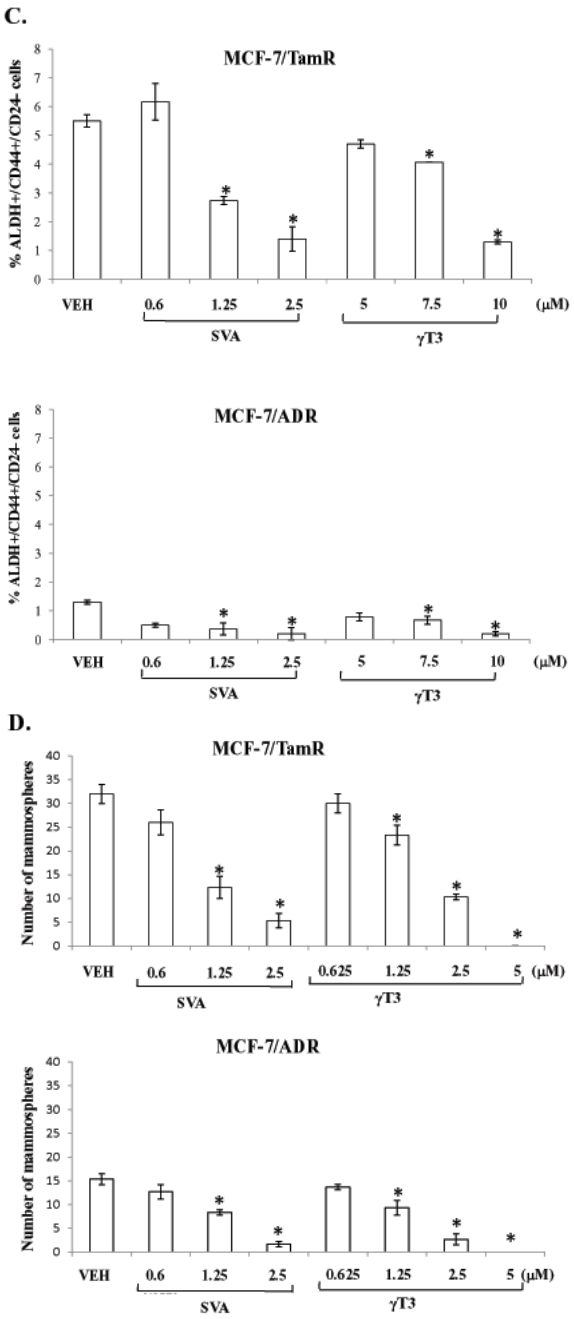


Figure 4.5: C and D

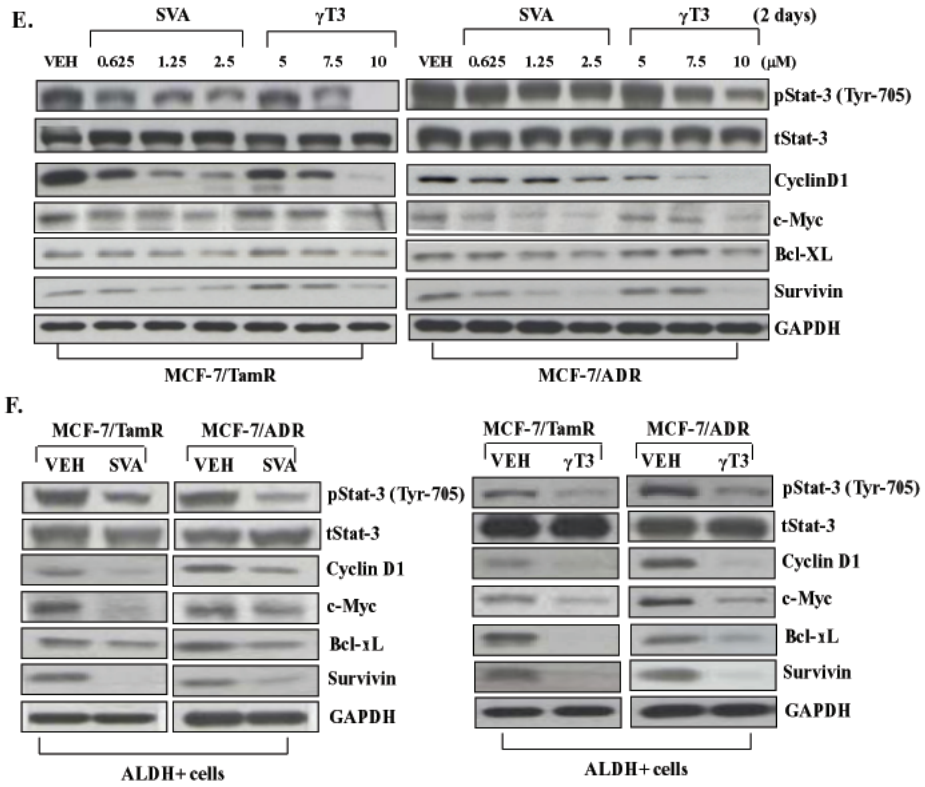


Figure 4.5: γT3 and SVA abolishes characteristics of TICs and suppresses pStat-3, c-Myc, Cyclin D1, Bcl-xL and Survivin protein expression in DR breast cancer cells.Unsorted cells were treated with indicated concentrations of γT3 and SVA for 2 days to determine ALDH+ population as detected by ALDH activity assay (A), to determine CD44+/CD24- population as detected by immune-staining assay (B) and ALDH+/CD44+/CD24- populations(C). ALDH+ populations were treated with indicated concentrations of γT3 and SVA for 7 days to determine the number of mammospheres as detected by mammospheres formation assay (D). Data are presented as means $\pm$ SD of three individual experiments. *significantly different from VEH, $p < 0.05$. Western blot analyses were performed to evaluate protein expression in unsorted cells treated with different concentrations of γT3 and SVA or VEH control for 2 days (E) and in ALDH+ enriched cells treated with γT3 at 7.5 μM and SVA at 0.625 μM or VEH control for 2 days (F). Data are representative of two or more independent experiments.

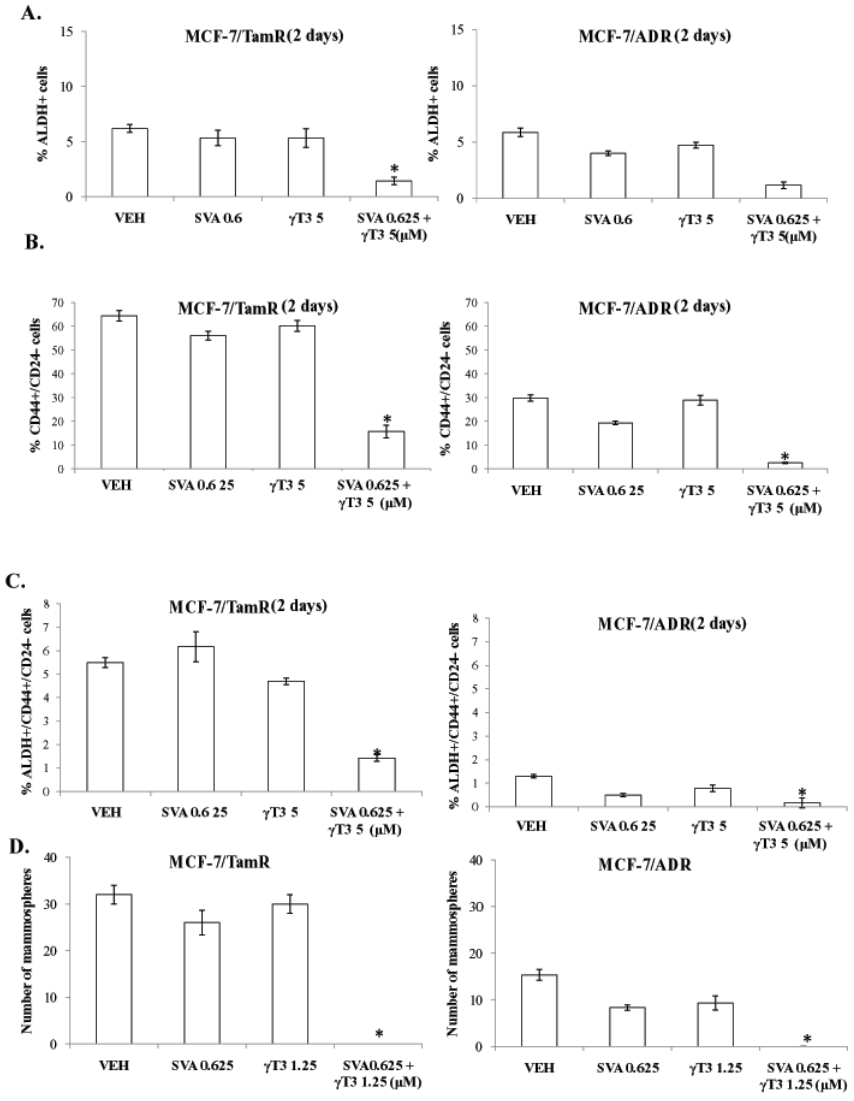
4.4.6 Combination of SVA and γT3 act cooperatively to reduce ALDH+ cells, and to suppress pStat-3(Tyr-705), Cyclin D1, c-Myc, Bcl-xL and Survivin protein levels in drug resistant breast cancer cells.

Since both SVA and γT3 as exhibit low bioavailability *in vivo* [177], combinations of low doses of SVA and γT3 that have been reported to be achievable in vivo [177] were studied in an effort to improve the anti-TIC efficacy, in comparison to single treatments. MCF-7/TamR and MCF-7/ADR cells were cultured with the combination of SVA (0.625 μM) and γT3 (5 μM) for 2 days. In comparison to single treatments, the combination of SVA plus γT3 significantly decreased ALDH+, CD44+/CD24- and ALDH+/CD44+/CD24- populations in both cell lines (Fig 4.6 A, B and C), and significantly reduced mammosphere formation (Fig 4.6 D). The combination of SVA and γT3 at low concentrations suppressed pStat-3(Tyr-705), Cyclin D1, and c-Myc protein levels in comparison to vehicle control and single treatments in both cell lines (Fig 4.6 E). Furthermore, SVA + γT3 treatment of ALDH+ cell populations from both cell lines acted cooperatively, in comparison to vehicle control, to suppress pStat-3(Tyr-705), CyclinD1, c-Myc, Bcl-xL and Survivin protein levels in ALDH+ population (Fig 4.6 F).

4.4.7 SVA and γT3 alone and in combination eliminate bulk drug resistant breast cancer cells via apoptosis.

Previous data showed that the combination of SVA + γT3 cooperatively induce apoptosis in mammary cancer cells [177], suggesting potential for this combination for breast cancer treatment. Since both of the drug resistant

cell lines used in these studies are resistant to chemotherapeutic agent-induced apoptosis; namely, ADR and cisplatin (data not shown) and tamoxifen [133], it is important to identify anti-cancer agents that can eliminate drug resistant bulk cancer cells. Therefore, the anticancer properties of SVA and γT3 alone and in combination were studied for ability to induce apoptosis in both drug resistant cell lines.



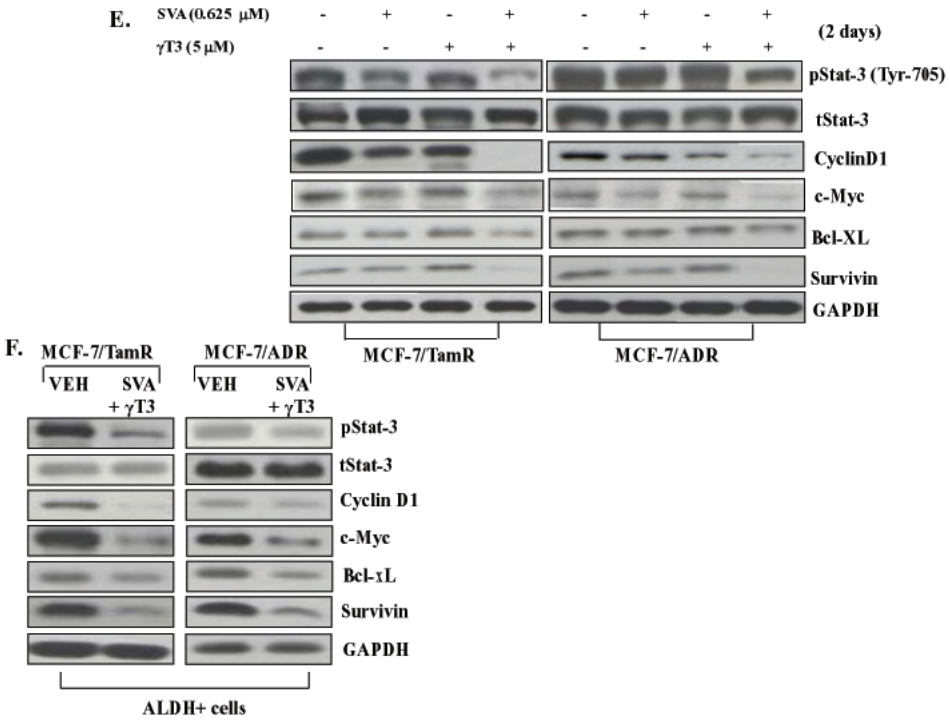
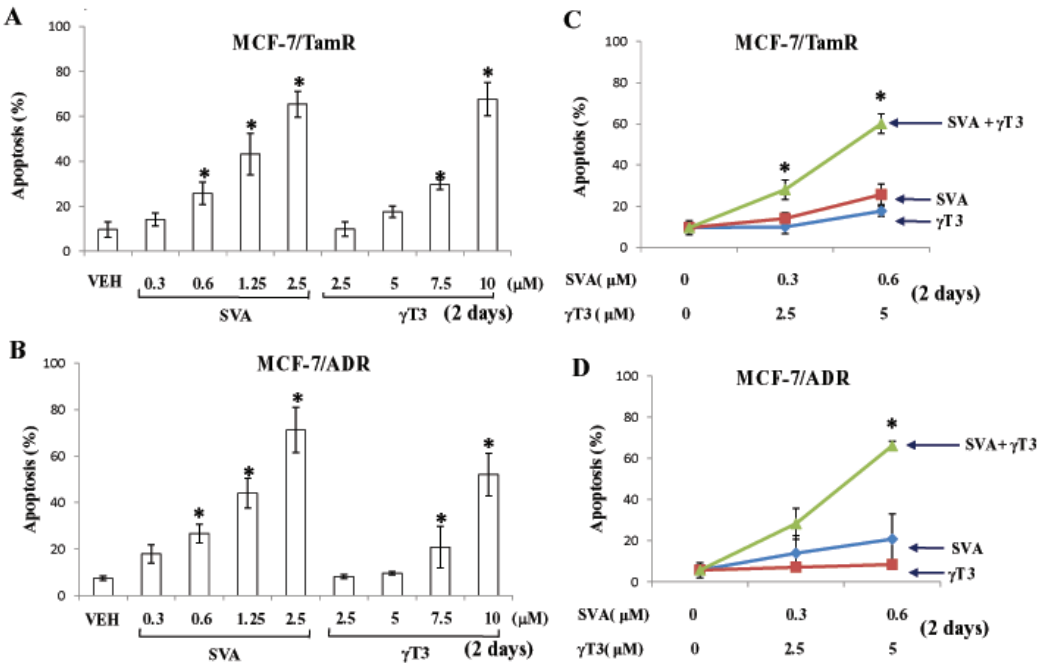


Figure 4.6: Combination of γT3 and SVA cooperatively eliminates TICs and suppress pStat-3, c-Myc, Cyclin D1, Bcl-xL and Survivin protein expression in DR breast cancer cells.Cells were treated with combination of γT3 and SVA for 2 days followed by ALDH activity assay (A), CD44/CD24 immuno-staining assay (B) and both assays (C). ALDH + cells were treated with combination of γT3 and SVA for 1 week for mammosphere formation assay (D). Western blot analyses were performed to evaluate protein expression in unsorted cells treated with γT3 at 5 μM and SVA at 0.625 μM or VEH control for 2 days and in ALDH+ enriched cells treated with γT3 at 2.5 μM and SVA at 0.3125 μM (E and F). Data from E and F are representative of two or more independent experiments. Data from A, B, C and D are presented as the mean $\pm$ S.D. of three independent experiments. *significantly reduced in comparison to Vehicle, $p < 0.05$.

Treatments of MCF-7/TamR and MCF-7/ADR cells with different concentrations of SVA or γT3 alone for 2 days significantly induced apoptosis in a dose response manner (Fig 4.7 A and B) and PARP cleavage, an indicator

of caspase dependent apoptosis (Fig 4.7 E). The combination of sub-apoptotic doses of SVA (0.625 μM) + γT3 (5 μM) significantly induced apoptosis, in comparison to SVA and γT3 alone, in both drug resistant cell lines (Fig 4.7 C, D and F). Furthermore, SVA and γT3 alone induced increased cleavage of caspases-8 and -9 (Fig 4.7 E). The combination of sub-apoptotic doses of SVA + γT3, in comparison to single treatments, enhanced cleavage of caspases 8 and 9 (Fig 4.7 F), suggesting involvement of death receptor- and mitochondria-mediated apoptotic pathway.



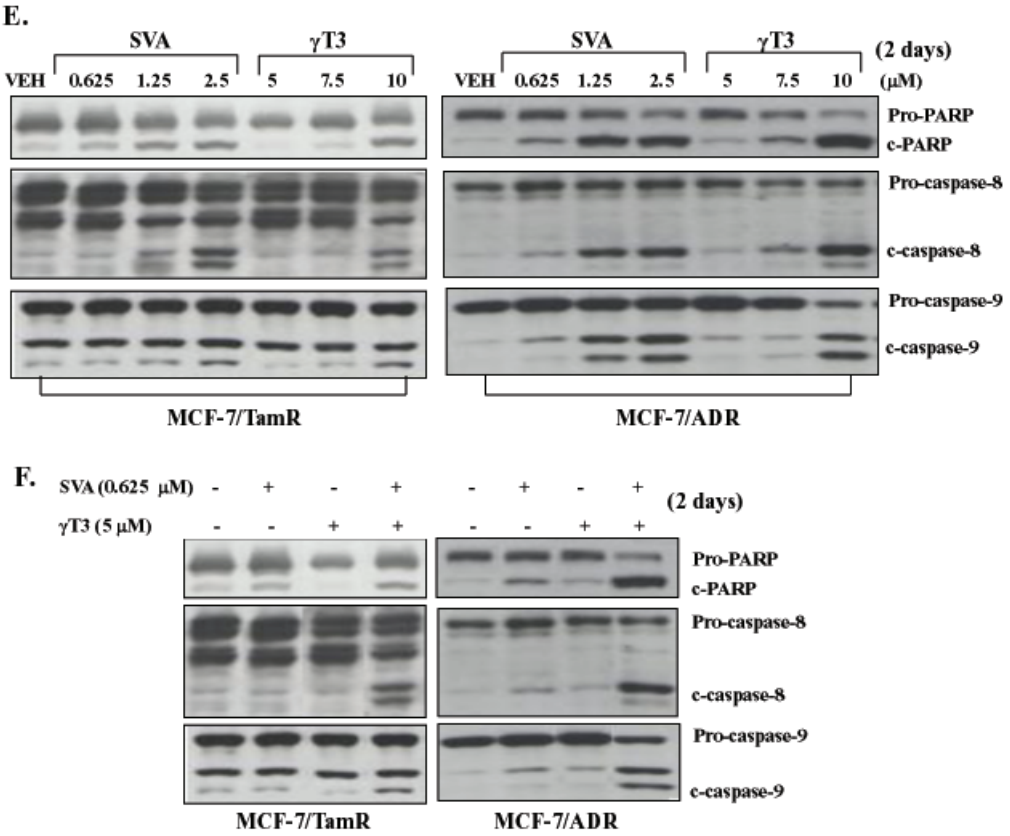


Figure 4.7: γT3 and SVA alone and in combination target bulk of drug resistant breast cancer cells by inducing apoptosis. Cells were treated with various concentrations of γT3 and SVA alone (A and B) or in combination of γT3 + SVA (C and D) for 2 days followed by Annexin V assay to determine apoptosis. Western blot analyses were performed to evaluate cleavage of PARP, caspase-8 and -9 (E and F). Data from E and F are representative of two or more independent experiments. Data from A, B, C and D data are presented as the mean ± S.D. of three independent experiments. *significantly reduced in comparison to Vehicle, $p < 0.05$. * significantly reduced in comparison to single treatments, $p < 0.05$.

4.4.8 SVA and γT3 alone and in combination activate JNK/CHOP/DR5 pro-apoptotic pathway.

Previous studies showed that SVA and γT3 alone induced apoptosis in human breast cancer cells, including MCF-7 cells, via activation of JNK, CHOP and DR5 [32] [submitted data]. Treatment of MCF-7/TamR and MCF-7/ADR cells with different concentrations of SVA or γT3 for 2 days increased pJNK (2/1), CHOP, DR5, and GRP78 protein levels (Fig 4.8 A). A combination of sub-apoptotic doses of SVA + γT3, in comparison with single treatments, acted cooperatively to enhance these pro-apoptotic proteins (Fig 4.8 B). These data suggest that both SVA and γT3 alone and in combination induce apoptosis in drug resistant cells via activation of JNK/CHOP/DR5 pro-apoptotic pathway.

4.5 Discussion

Both SVA and γT3 alone and in combination exhibit anticancer actions *in vitro* and *in vivo* [52–54, 124]. In this study we evaluated their anticancer action on eliminating TICs in tamoxifen and adriamycin resistant human breast cancer cell lines. The novel findings of this study are: (i) Like MCF-7/ADR cells [132], MCF-7/TamR cells are enriched in TICs in comparison to the parental cell line, (ii) Both drug-resistant cell lines express higher levels of pStat-3, c-Myc, CyclinD1, Bcl-xL and Survivin than parental lines and these pro-proliferation/survival factors are concentrated in TIC enriched cells, (iii) expression of phosphorylated Stat-3 contributes to increased numbers of TICs in drug resistant cell lines, (iv) SVA and γT3 eliminated TICs as well as sup-

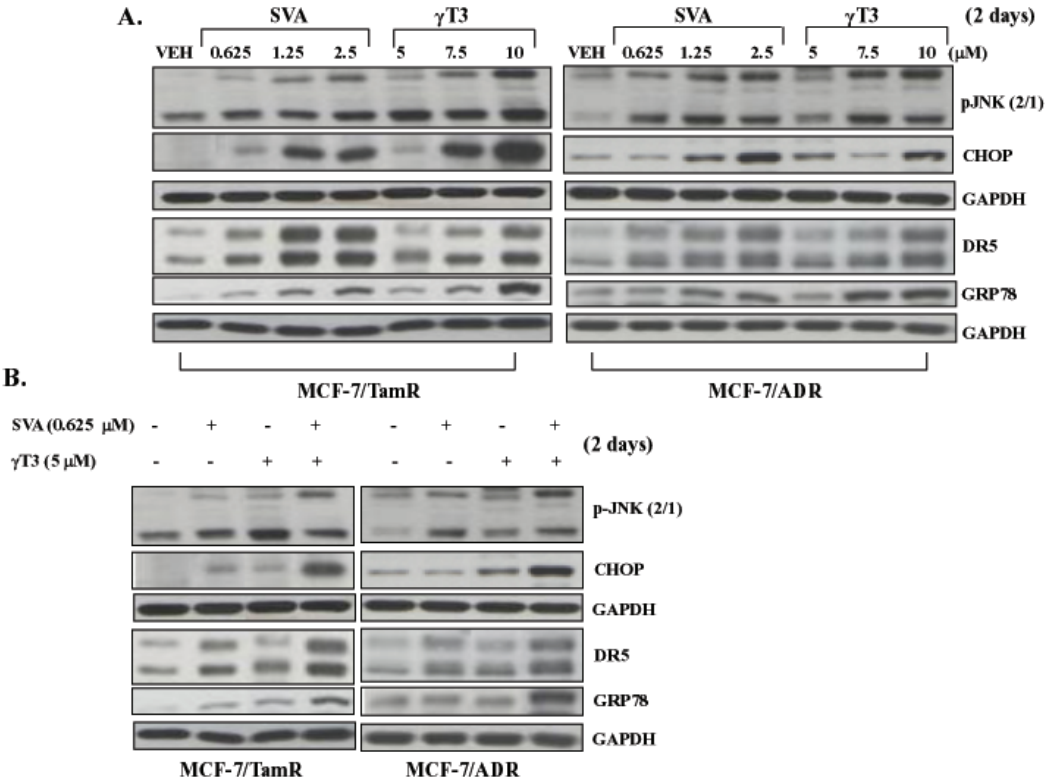


Figure 4.8: γT3 and SVA alone and in combination induce activation of JNK/CHOP/DR5 pro-apoptotic pathway.Western blot analyses were performed to evaluate protein expression. Data are representative of two or more independent experiments.

pressed protein expression of Stat-3, c-Myc, CyclinD1, Bcl-xL and Survivin in both cancer bulk and TIC cell populations, and (v) both SVA and γT3 alone induce apoptosis in these two drug resistant cell lines which is associated with activation of the JNK/CHOP/DR5 pro-apoptotic pathway and the combination of SVA + γT3 cooperatively enhanced these anticancer events. Taken together, our data demonstrated that SVA and γT3 alone and in combination at pharmacologically achievable doses exhibit the ability to target both TIC-enriched and TIC depleted cell populations in drug resistant breast cancer cell lines,

suggesting that the combination of SVA and γT3 may be a potential strategy for prevention and treatment of drug resistant breast cancer by targeting both TIC-enriched and non-TIC cells. Data also provide evidence that Stat-3 may be a novel target for prevention and treatment of drug resistant breast cancer via enhanced elimination of TIC-enriched populations. Based on the cancer stem cell concept, targeting TICs provide a new hope for eradicating cancers, including breast cancer. Accumulating evidence support the observations that traditional cancer therapies such as chemotherapeutic agents and radiation therapy specifically target non-TICs/bulk of the tumor cells, leaving behind the more resistant TIC populations which can generate tumors. Several lines of *in vitro* and textitin vivo data show that conventional therapies enrich for TICs [112, 116, 186] thus, leading to tumor recurrence, drug resistance and metastasis. These may explain why current therapies are incapable of eradicating cancer. Based on the cancer stem cell concept, targeting TICs holds promise for prevention and treatment of drug resistance.cancer statistics Therefore, the finding that drug resistant cells contain a higher percentage of TIC-enriched cells supports the TIC concept that drug resistant cancers become more aggressive, metastatic and treatment resistant due to selection of TIC population. Previously, Calcagno AM, et al. reported that in comparison to parental cells, MCF-7/ADR cells (the same cell line used in this study) were highly invasive, formed mammospheres and contain higher percentage of CD44+/CD24- cells *in vitro* which resulted in higher tumorigenicity textitin vivo [132]. Their data indicated that cells possessing cancer stem cell characteristics were enriched in the MCF-7/ADR cell line in comparison to the parental cell line [132].

Although acquired MCF-7/TamR cells have been shown to exhibit an EMT phenotype [112, 173], there are no published data supporting that TICs are enriched in TamR cells. Calcagno AM, et al. also reported that the MCF-7/ADR cells were enriched in ALDH+ cells but it was not confirmed to be a TIC marker like seen in SUM159 human breast cancer cells [109]. Here, our data demonstrate that both resistant cell lines contain higher levels of ALDH+ cells, and that cells enriched for ALDH+ exhibit cancer stem cell characteristics; namely, formation of greater numbers of mammospheres (Figure 2A) in comparison with ALDH- populations in both drug resistant cell lines, and ability to differentiate into ALDH- cells (Table1). In addition, both cell lines contain higher levels of CD44+/CD24- populations. CD44+/CD24- has been identified as a TIC marker in multiple breast cancer cell lines and tumors [113] but they do not always represent a true TIC population. However the CD44+/CD24- phenotype has been challenged as a biomarker for TICs by some [187]. CD44+/CD24- populations have been studied as a TIC marker in regular MCF-7 cells [188] and Calcagno AM, et al. reported that the CD44+/CD24- population in MCF-7/ADR exhibits TIC characteristics such as self-renewal and tumor initiation [132]. Croker AK, et al. reported that the ALDH+/CD44+/CD24- population is more enriched for TIC as well as chemotherapy and radiation resistance in MDA-MB-231 cells [180, 187]. Based on these studies, we examined the expression of ALDH+/CD44+/CD24- populations in both drug sensitive and drug resistant cell lines. In comparison with their parental cell lines, both MCF-7/TamR and MCF-7/ADR cell lines not only expressed higher percentage of ALDH+, CD44+/CD24-, but also

ALDH+/CD44+/CD24- populations. Taken together, our data demonstrate that MCF-7/TamR and MCF-7/ADR contain higher levels of TIC-enriched populations. TICs are regulated by multiple mediators, including Wnt/beta-catenin, PI3K/PTEN/mTOR/AKT, Notch, Hedgehog, NF- κB, Her-2 and Stat-3 [107, 182]. However, the factors that regulate TICs in drug-resistant breast cancer have not been well addressed. Over-expression of growth factor receptor signaling pathway in MCF-7/TamR cells is well documented [133]. Data also show that NF- κB, Stat-3, Akt, and ERK are highly expressed in this cell line [133][unpublished data]. On the other hand, MCF-7/ADR cell line has been reported to express high levels of multiple drug resistant (MDR) and NF κB proteins [189]. Furthermore, we observed that pERK, pStat-3 and pIκB, but not pAkt, are over-expressed in MCF-7/ADR compared to the parental cell line (Fig 3A, unpublished data). By characterizing the molecular profile we found that overexpression of pStat-3 (Tyr-705), c-Myc, CyclinD1, Bcl-xL and Survivin is a common event for both drug resistant cell lines. Importantly, these signaling events are more highly elevated in TIC enriched populations in comparison with TIC-depleted and unsorted populations. Stat-3 has been reported to mediate TICs in MCF-7 cells [182] and contributes to maintenance of TIC *in vivo* as well [122]. In agreement with these data, our data show that knockdown of Stat-3 using Stat-3 inhibitor eliminated TIC-enriched populations in both drug resistant cell lines and thus support the role of Stat-3 in regulating TICs of drug resistant breast cancer cells. These important findings suggest that Stat-3 may be an important target for eliminating TICs in drug resistant human breast cancers irrespective of the origin of resistance. c-Myc,

CyclinD1, Bcl-xL and Survivin are established stem cell mediators [183, 184] and can be regulated by different pathways, such as growth factor receptor mediated Akt, NF-κB and Stat3 [190–192]. Our data showing that a Stat-3 inhibitor reduced c-Myc, CyclinD1, Bcl-xL and Survivin protein expression, suggests that Stat-3 is an upstream mediator of these factors in these two drug resistant cell lines. One of the novel findings in this study is that SVA and γT3 alone or in combination eliminate TIC-enriched population as demonstrated by treatment: (i) elimination of ALDH+ population, (ii) reduction in CD44+/CD24- cell numbers as well as ALDH+/CD44+/CD24- numbers, and (iii) inhibition of mammosphere formation by ALDH+ population. Furthermore, our data show that SVA and γT3 alone or in combination inhibited pStat-3 as well as c-Myc and CyclinD1 protein expression. Since our data suggest that Stat-3 is involved in regulating TICs in both drug resistant cell lines, we hypothesized that downregulation of Stat-3 and its downstream mediators c-Myc, CyclinD1, Bcl-xL and Survivin contribute to the ability of the treatments to eliminate TICs in drug resistant cell lines. Stat-3 is a transcription factor and has been reported to be regulated mainly by JAK and Src mediated activation of NF-κB/IL-6 [193]. Also, SHP-1, a tyrosine phosphatase, can negatively regulate Stat-3 via dephosphorylation of pStat-3 at Tyr-705 [39]. Both SVA and γT3 alone have been reported to downregulate pStat-3 [111, 179]. γT3 has been reported to downregulate pStat-3 via upregulation of SHP-1 [179]. Our unpublished data show that both SVA and γT3 alone and in combination induced increased levels of SHP-1 (Data not shown). Since both SVA and γT3 have been reported to downregulate NF-κB

[52, 185], it is possible that both downregulation of NF-κB and upregulation of SHP-1 contribute to SVA and γT3 suppression of Stat-3. Proof of NF-κB and SHP-1 involvement remain to be tested. Pro-apoptotic properties of SVA and γT3 alone and in combination have been reported in different cancer types, including breast cancer cells [32, 50–54, 177, 194]. However, to the best of our knowledge, this is the first report that SVA and γT3 alone or in combination induce apoptosis in drug resistant cell lines. It is important to develop strategies to effectively kill drug resistant cancer cells since overtime they develop cross-resistance to multiple anticancer treatments [2]. For example; MCF-7/ADR cells are resistant to tamoxifen due to down regulation of estrogen receptor expression and cells exhibit resistance to cisplatin (unpublished data) [133]. Likewise MCF-7/TamR cells exhibit greater resistance both to doxorubicin and cisplatin possibly due to the high expression of multiple anti-apoptotic factors [32][unpublished data]. Furthermore, previous data show that JNK/CHOP/DR5 are activated and involved in both SVA- and γT3-induced apoptosis in MCF-7 cells [32] [submitted manuscript]. In agreement with these data SVA and γT3 alone activated JNK, CHOP and DR5 pro-apoptotic mediators in these drug resistant breast cancer cells, suggesting that JNK/CHOP/DR5 pro-apoptotic pathway contribute to SVA- and γT3- induced apoptosis. Although the combination of SVA + γT3 has been reported previously to cooperatively induce apoptosis, the underlining mechanism was not addressed [177]. Here, we report that the combination of SVA + γT3 cooperatively induces activation of JNK/CHOP/DR5. Despite the fact that SVA and γT3 alone exhibit both anti-TIC and anti non-TIC properties,

the combination effect of lower doses of SVA + γT3 on TICs and non-TICs elimination may be more clinically relevant based on bioavailability and less toxicity. For example, the *in vivo* concentrations of SVA of 2 μM have been associated with multiple toxic side effects [138]. Hence, the combination strategy provides an opportunity to use less toxic concentrations without sacrificing treatment efficacy. In summary, our data demonstrate that two different drug resistant human breast cancer cell lines contain higher levels of TIC enriched populations that in part are maintained via highly expressed phosphorylated Stat-3 and its downstream mediators c-Myc, CyclinD1, Bcl-xL and Survivin, and that SVA and γT3 target TICs via suppression of phosphorylated Stat-3 and its downstream mediators c-Myc, CyclinD1, Bcl-xL and Survivin. Data provide new insights into how TICs are regulated in drug resistant breast cancer cells and suggest the potential for the combination of SVA + γT3 as a novel regimen for prevention and treatment of drug resistant breast cancer via targeting TICs and bulk tumor cells. Data also suggest that phosphorylated Stat-3 is a potential target for elimination of TICs in drug resistant breast cancer cells. These *in vitro* data provide strong rationale for further *in vivo* study.

Chapter 5

Summary and future directions

5.1 Conclusion

γT, γT3 and SVA have been reported to display anticancer properties against multiple types of cancer both in cell culture and in pre-clinical animal models, providing support for the use of γT, γT3 and SVA as anticancer therapeutics yet the mechanisms involved in these actions remain elusive. Studies here, focused on identifying mechanisms involved in anticancer effects mediated by two natural forms of vitamin E γT and γT3; as well as, simvastatin in human breast cancer cells. Briefly, γT and γT3 were observed to induce apoptosis via activation of *de novo* ceramide pathways leading to upregulation of proapoptotic JNK/CHOP/DR5 and downregulation of c-FLIP, Bcl-2 and Survivin. SVA mediated apoptosis was observed to involve activation of proapoptotic JNK/CHOP/DR5 axis where JNK/CHOP were in part responsible for SVA mediated downregulation of c-FLIP and Survivin. Furthermore, γT3 and SVA alone and in combination were demonstrated to target TIC populations in drug resistant breast cancer cells. In summary, these studies improved our present understanding and provide key insights that will benefit future studies aimed at using these agents as therapeutics for breast cancer in human.

Chapter 2 studies determined downstream mediators of apoptosis in SVA treated human breast cancer cells. Work done by Koyuturk M et al, 2007 demonstrated that JNK is involved in SVA induced apoptosis but factors and process from JNK activation to actual induction of apoptosis were unknown. Work here shows that SVA induced prolonged phosphorylation of JNK leading to apoptosis via downstream activation of CHOP and DR5. Simultaneously, SVA-induced apoptosis is accompanied by downregulation of antiapoptotic factors c-FLIP and Survivin which are in part dependent on JNK/CHOP/DR5 proapoptotic axis. Exogeneously added mevalonate or geranylgeranyl pyrophosphate (GGPP) blocked SVA-induced activation of JNK/CHOP mediated apoptosis and downregulation of c-FLIP and Survivin. These novel findings provide insights into the critical events involved in SVA-induced apoptosis in human breast cancer cells.

Chapter 3 compared events common to both γT and γT3 induced apoptosis. Studies also helped identify the upstream mediators of γT induced activation of DR5 in human breast cancer cells. Similar to γT3, γT induced DR5 activation was observed to involve upstream activation of JNK and CHOP and was accompanied by activation of endoplasmic reticulum stress. Another finding here is the dependence of downregulation of antiapoptotic factors by γT on JNK/CHOP/DR5 proapoptotic loop. In addition, both forms of vitamin E were observed to initiate *de novo* ceramide synthesis prior to activation JNK/CHOP/DR5, apoptosis, and downregulation of antiapoptotic factors. These finding demonstrate that γT induces apoptosis via activation of similar upstream mediators like γT3 and apoptosis induced by both forms of vitamin

E involve activation of the *de novo* ceramide pathway.

Chapter 4 characterized the molecular differences between drug- sensitive and -resistant human breast cancer cells. Increased expression of signal transducer and activator of transcription 3 (Stat3) has been correlated with invasive malignant tissue compared to surrounding normal tissue in breast cancer (Diaz et al 2006) but there is no evidence in the literature for the role of Stat3 in drug resistance and regulation of TICs in drug resistant breast cancer [195]. Work here shows that both forms of drug resistant breast cancer cells express higher levels of TIC populations and phosphorylated Stat3 as well as its downstream mediators c-Myc, cyclinD1, Bcl-xL and Survivin compared to their drug sensitive parental counterparts. Inhibition of Stat3, demonstrated a role for Stat3 in maintenance of TICs in drug resistant breast cancer. Moreover, γT3, SVA and combination of γT3+SVA were observed to target the over expressed Stat3 pathway and TICs in drug resistant cells. Hence, development of drug resistance in human breast cancer is associated with increased expression of Stat3 and TICs which can be counteracted by γT3, SVA or the combination of γT3+SVA treatments.

5.2 Future Directions

During the course of this study, multiple questions were raised which need to be addressed in future studies to gain a more complete understanding of mechanisms involved in γT, γT3 and SVA mediated anticancer effects. Firstly, previous studies have shown that γT induced activation of Peroxisome proliferator-activated receptor gamma (PPARγ) receptors which may play an

important role in apoptosis and proliferation but there is no evidence in the literature investigating the interrelationship between induction of PPARγ activation with *de novo* ceramide synthesis in γT treatments [196]. Although PPARγ has been shown to activated by C2-ceramide in human colon adenocarcinoma, there is no evidence in literature to show if these pathways are connected[197], further studies will have to be carried out to determine if these events occur in a sequence: namely does there exists any cross-talk between these two pathways? Or is the activation of these pathways independent simultaneously occurring events in γT induced apoptosis?

Secondly, it is necessary to test the tumor initiating property of ALDH enriched versus ALDH depleted cells *in vivo* in NOD/SCID mice. Thus, providing proof of principle for existence of TIC populations in ALDH enriched subpopulation and lending further support to the already obtained *in vitro* data. Furthermore the effect of orally administered SVA and γT3 in combination for its ability to target TIC populations has to be tested *in vivo* in NOD/SCID mice.

Thirdly, there is evidence in the literature that γT3 induced apoptosis is accompanied by activation of protein tyrosine phosphatase SHP-1 and suppression of Stat3 and NFκB pathways [38, 39]. It would be of interest to determine if activation of JNK/CHOP/DR5 axis is responsible for downregulation of transcription factors, Stat3 and NFκB or if these are independent events that occur during γT3 treatment. Such studies help in the development of multiple biomarkers for identification of γT3 treatment efficacy to be used in clinical assessments.

Fourthly, Stat3 has been shown in the literature to be regulated by SHP-1 and NFκB [39, 193]. Furthermore, γT3 and SVA have been shown in the literature to downregulate NFκB in human breast cancer cells [52, 185]. In this work, for the first time, a role for Stat3 in the development of drug resistance and TICs was demonstrated. Also, SVA was observed to enhance SHP-1 in a dose dependent manner and drug resistant cells expressed lower levels of SHP-1 protein compared to the parental cells (Figure 5.1 A and B). Hence, there is a need to determine if SHP-1+NFκB or if one or the other of these factors can regulate Stat3 in drug resistant breast cancer and if so do they play a role in SVA or γT3+SVA mediated anti-TIC effects.

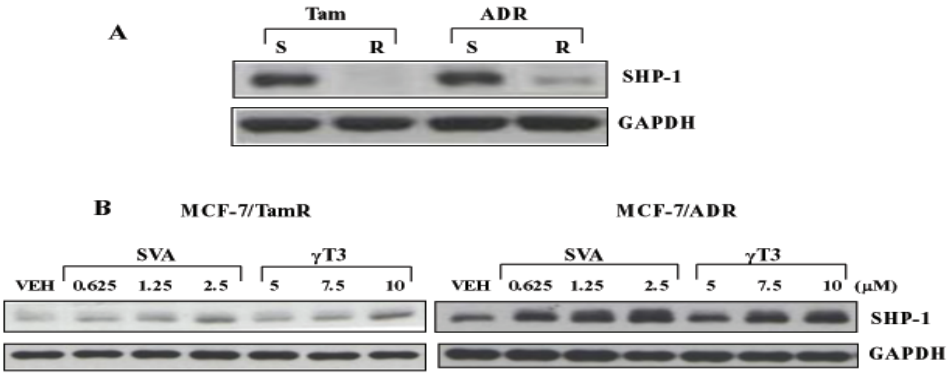


Figure 5.1: SHP-1 regulation in drug resistant cells.

SHP-1 regulation in drug resistant cells.Western blot analyses of MCF-7/TamS, MCF-7/TamR, MCF-7 and MCF-7/ADR untreated or treated with indicated concentrations of γT3 and SVA for 48 hours. GAPDH was used as loading control. Data representative of at least three independent experiments.

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