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Anti-tumor actions of Vitamin E analog α-TEA alone and in combinations in human breast cancer cells

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Anti-tumor actions of Vitamin E analog α-TEA alone and in combinations in human breast cancer cells

by

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Dedication

To my husband, Ashu, for his unconditional love and for inspiring me to follow my dreams. Thank you for being my best friend.

And to my parents for their never ending love and care and for having total faith in me.

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First and foremost, I would like to thank Dr. Bob G Sanders and Dr. Kimberly Kline for giving me the incredible opportunity to learn and grow in their lab as well as for their continuous encouragement and support. Their very special ability to give me the freedom to pursue my ideas independently while also providing guidance when needed is a rare gift and one that I very much appreciate and value highly.

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Finally, I would like to thank my cheer leaders: my husband, my parents, my sister and my brother; for always being there for me.

Anti-tumor actions of Vitamin E analog α-TEA alone and in combinations in human breast cancer cells

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Breast cancer is the second leading cause of mortality among women in the US. A contributing factor to such dire statistics is that conventional therapies are all too often compromised due to tumor relapse. Clearly there is an urgent need for agents that can circumvent resistance when combined with conventional therapies. RRR-α-tocopherol ether-linked acetic acid analog (α-TEA), a small bioactive lipid, exhibits *in vitro* and *in vivo* anticancer actions in a variety of cancers, including breast, prostate, and ovarian with little or no effect on normal cells and tissues, which potentially makes it an ideal chemotherapeutic agent. My studies investigated the anticancer actions of α-TEA alone and in combination with therapeutic agents using human breast cancer cell lines. Data show that:

(i) Endoplasmic reticulum (ER) stress plays an important role in α-TEA induced apoptosis by enhancing DR5/caspase-8 pro-apoptotic signaling and suppressing anti-apoptotic factors c-FLIP and Bcl-2 via ER stress mediated JNK/CHOP/DR5/caspase-8 signaling,

(ii) α-TEA plus tamoxifen act cooperatively to circumvent acquired and de novo tamoxifen resistance, resulting in cancer cell death by apoptosis. Mechanistically, the circumvention of tamoxifen resistance involved induction of DR5/caspase-8 pro-apoptotic mediators and suppression of anti-apoptotic factors c-FLIP and Bcl-2 via ER stress mediated JNK/CHOP/DR5/caspase-8 signaling.

(iii) α-TEA alone or with tamoxifen circumvents tamoxifen resistance via disruption of membrane cholesterol rich lipid raft microdomains. Cholesterol blocked the ability of α-TEA + tamoxifen to circumvent tamoxifen resistance.

(iv) α-TEA in combination with PI3K, MEK or mTOR inhibitors acted cooperatively to induce apoptosis, by down-regulation of IRS-1/PI3K mediators via JNK.

(v) α-TEA plus doxorubicin or cisplatin enhanced apoptosis in p53 mutant human breast cancer cells via targeting p53-inducible genes in a p73-dependent manner; namely, via up-regulation of death receptor-5 (DR5), CD95/APO-1 (Fas), Bax and Noxa, as well as down-regulation of anti-apoptotic mediator Bcl-2. Data showed that p73 responses were downstream of c-Abl, JNK and Yap.

(vi) FASN inhibitor alone or with Tamoxifen or α-TEA circumvents tamoxifen resistance, thereby, providing novel strategies for restoring tamoxifen sensitivity to tamoxifen resistant cancers.

In summary data show, α-TEA alone and in combination with multiple clinically-relevant anticancer agents is a promising anticancer agent.

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Chapter 1: Introduction

1.1 Breast Cancer

There have been significant advances in medicine and understanding of human disorders and diseases, especially during the past two decades. Based on epidemiological data, there have been tremendous reductions in mortality from cardiovascular and infectious diseases. Despite significant advances achieved in understanding cancer progression, targeted therapies, and prevention of cancer, reduction and elimination of cancers remain elusive [Jemal et al., 2006; Aggarwal et al., 2006]. Cancer is characterized by uncontrolled cellular growth and metastasis. Three factors are attributed to cause cancers: environmental factors (chemicals, radiation, infectious organisms), genetics (inherited mutations, hormones, immune conditions) and lifestyle choices (diet, obesity, tobacco).

Breast cancer is the second leading cause of death in women after lung cancer. It is responsible for nearly 40,000 deaths each year in the United States [http://www.cancer.gov/cancertopics/types/breast]. Cancer forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). It occurs in both men and women, although male breast cancer is rare. Well recognized risk factors for non-hereditary breast cancers include: age, sex, use of birth control pills or hormone replacement therapy, high fat diet, alcohol, obesity, as well as age at menarche and menopause, menstrual

irregularity, and age at first and last childbirth. Parity and breast feeding have also been linked to breast carcinogenesis [Okobia et al., 2005]. Breast cancer may also be caused by inherited gene mutations. Hereditary breast cancers account for approximately 5% to 10% of all breast cancers. Specific hereditary predispositions for breast cancer, include inheriting mutations in either the BRCA1 or BRCA2 genes [http://www.cancer.gov/bcrisktool/breast-cancer-risk.aspx].

1.2 Current therapies

Stage of the breast cancer determines the type of treatment, but often includes chemotherapy, endocrine therapy, surgery, and radiation therapy. Recently, targeted therapy such as Herceptin (trastuzumab) has also become part of standard treatment for tumors expressing Her-2 receptors [http://www.herceptin.com]. Preclinical experiments and clinical trials are currently underway to evaluate additional targeted therapies and to determine how to best use these targeted agents in combination with each other and with standard therapies.

1.2.1 Endocrine therapy (ET)

It has long been established that estrogen is involved in the pathogenesis of breast carcinoma, and that it sustains growth of breast cancer that express estrogen receptor (ER). Approximately 75% of all breast tumors rely on estrogen and are referred as estrogen dependent. Tumor cells expressing estrogen receptors (ER) are called ER positive (ER+) [Susan G. Komen for the Cure: Breast Cancer Facts, 2009]. The estrogen dependency of breast cancer represents a unique feature of the disease that can be manipulated to effectively control growth and prevent tumor development. The current strategy for treatment of hormone dependent breast cancer is to block the action of estrogen on tumor cells via three possibilities: 1) inhibiting the binding of estrogen to ER by using antiestrogen such as tamoxifen, 2) preventing the synthesis of estrogen via using an aromatase

inhibitor such as anastrozole, 3) downregulating ER protein level using pure antiestrogen such as fulvestrant [Forbes et al., 2008].

Tamoxifen, the first clinically relevant selective estrogen receptor modulator (SERM), acts as an antagonist in the breast and is available as therapy for postmenopausal women with early stage ER positive breast cancer. Data from adjuvant breast cancer trials have shown that five years of tamoxifen therapy suppresses the recurrence of breast cancer and reduces the incidence of contralateral second primary breast tumors by 50% [Fabian et al., 2007]. Tamoxifen has also been known to have beneficial effects as a chemopreventive agent. Based on data showing that tamoxifen reduced the chances of developing breast cancer by 50% in high-risk pre and post-menopausal women, in 1999 tamoxifen became the first endocrine therapy to be approved by the FDA to prevent breast cancer [Massarweh et al., 2006; Masri et al., 2008].

Tamoxifen has estrogenic actions in endometrial tissue, adipose tissue and bone and anti-estrogenic actions in breast tissue. Tamoxifen binds to and antagonizes ER and has been the mainstay of endocrine therapy in both early and advanced breast cancer patients for almost three decades [Fabian et al., 2007].

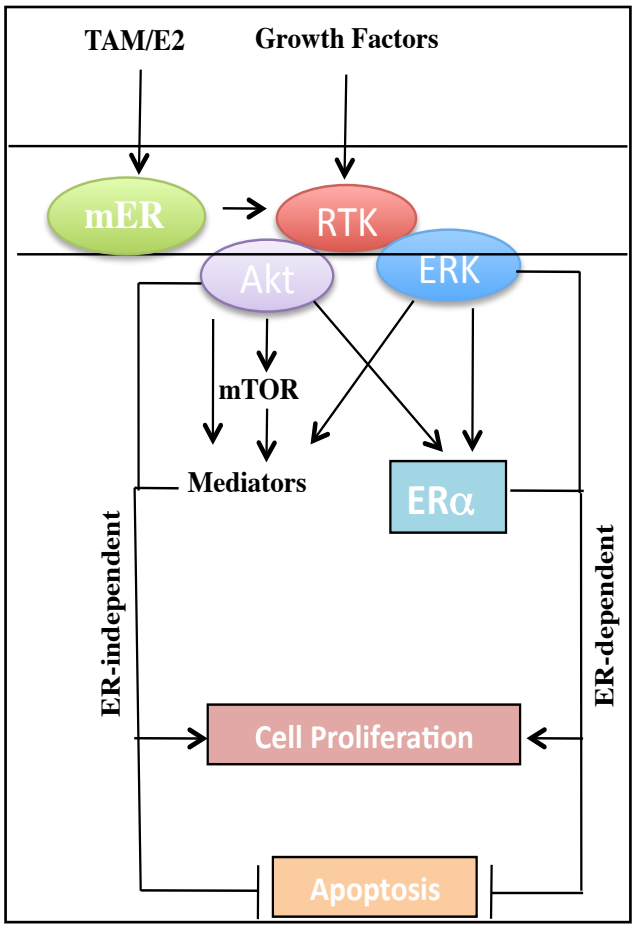


Figure 1 summarizes deranged cellular protein expression and dysregulated cell-signaling pathways that produce ET resistance. In ET resistant cells, growth factor receptors (RTKs: Her-1, Her-2 and IGFR) are over-expressed and constitutively activate their downstream mediators Akt and ERK via both E_2-dependent (ligand dependent) and E_2-independent pathways. In the ligand dependent pathway, E_2 or TAM activates Akt/ERK via binding with mER, leading to crosstalk between mER and RTK. In this event, TAM acts as ER agonist due to over-expressed RTKs. Activation of Akt and ERK activate ER-α via phoshorylation, leading to its translocation into the nucleus and activation of ER-dependent genes (termed ER non-genomic pathways), which drive cancer cell proliferation and survival. Akt, and ERK also activate ER-independent signal transduction mediators that are required for cell proliferation and survival. Thus, activation of RTKs and their downstream mediators Akt and ERK; namely, RTK/Akt/ERK complex (Her-1, Her-2, IGFR-1, Akt and ERK) via ligand-independent pathway represent major mechanisms promoting ET resistance.

Unfortunately, 40-50% of all ER+ breast cancer patients will either not respond to ET (i.e. exhibit de novo resistance) or after initial successful response to treatment will suffer cancer recurrence that is ET resistant (i.e. exhibit acquired resistance) [Massarweh et al., 2006]. Mechanisms of resistance have been identified for both de novo and acquired TAM resistance. Since expression of ERα is the main predictor of response to ET, lack of expression of ER is one of the mechanisms of de novo resistance [Masri et al., 2008]. Mutations of ERα might also affect the response to anti-estrogens. The transcriptional regulatory activity of ER is significantly modulated by both co-activators and co-repressors. Data show that high levels of co-activators enhance agonistic activity of tamoxifen [Musgrove et al., 2009]. Similarly, reduced levels of co-repressors are associated with resistance to tamoxifen [Hurvitz et al., 2008].

Mechanisms of resistance have been identified for both de novo and acquired ET resistance. Resistant phenotypes include loss of ER, functionally defective ER, lack of drug potency due to metabolic degradation, and activation of alternative growth signaling pathways [Musgrove et al., 2009]. Of the many possibilities, aberrant over-expression of growth factor signaling pathways are implicated as important contributors to both acquired and de novo ET (TAM and AI) resistance [Riggins et al., 2007]. In ET resistant cells, over-expression of RTK's; such as Her-1, Her-2 or IGF1R, activate Akt and ERK, leading to cell resistance to ET via promoting cell proliferation and inhibiting apoptosis [Arpino

et al., 2008; Knowlden et al., 2005]. This can occur in an estrogen-independent or TAM-stimulated manner leading to up regulation of both ER-a dependent genes and ER-a independent mediators that are required for cell proliferation and survival [Marquez et al., 2006] (Figure 1). Crosstalk between RTK's & mERα, and RTKs with each other play important roles in this process [Patra, 2008].

Based on a number of reasons, including side effects and costs, tamoxifen will remain the better choice for some U.S. women and based on cost alone tamoxifen will continue to be the primary form of endocrine therapy for women in under-developed nations. Thus, there is still a need to understand how resistance can be circumvented for tamoxifen.

1.2.2 Chemotherapy

Successful treatment of triple negative (estrogen receptor negative, progesterone receptor negative and Her-2 negative) breast cancers (TNBC) that are also p53 mutant remain elusive. Both Doxorubicin (DOXO) and cisplatin (CDDP) are DNA damaging drugs that exert their anticancer actions via inhibition of cellular proliferation and induction of cell death by apoptosis. Tumor suppressor gene p53 plays a central role in the anticancer actions of DNA damaging agents [Yoshida et al., 2010; Mendoza-Rodríguez et al., 2001]. In cancer cells that express wild type p53, p73 has been reported to cooperate with p53 to induce apoptosis [Flores et al., 2002]; whereas, in p53 mutant cancer cells

p73 has been reported to induce apoptosis via activation of p53 apoptotic signaling mediators [Zhu et al., 2001]. Recent data show that p73 is up-regulated in response to a subset of DNA-damaging agents, including DOXO, CDDP, camptothecin and etoposide [Moll et al., 2004; Leong et al., 2007]. DOXO and CDDP induction of p73 mediated apoptosis has been reported in both p53 wildtype and mutant p53 human breast cancer cells [Irwin et al., 2003; Vayssade et al., 2005].

Despite the treatment success observed with DOXO and CDDP, severe nonspecific cytotoxicity (cardiomyopathy) and drug resistance are major limiting factors in their clinical application [Ferreira et al., 2008]. Significant efforts have been directed toward developing approaches toward decreasing systemic cytotoxic effects of doxorubicin while enhancing its therapeutic efficacy [Broxterman et al., 2009]. One of the strategies is combination therapy [Riganti et al., 2006; Indermaur et al., 2010].

p73 and Apoptosis

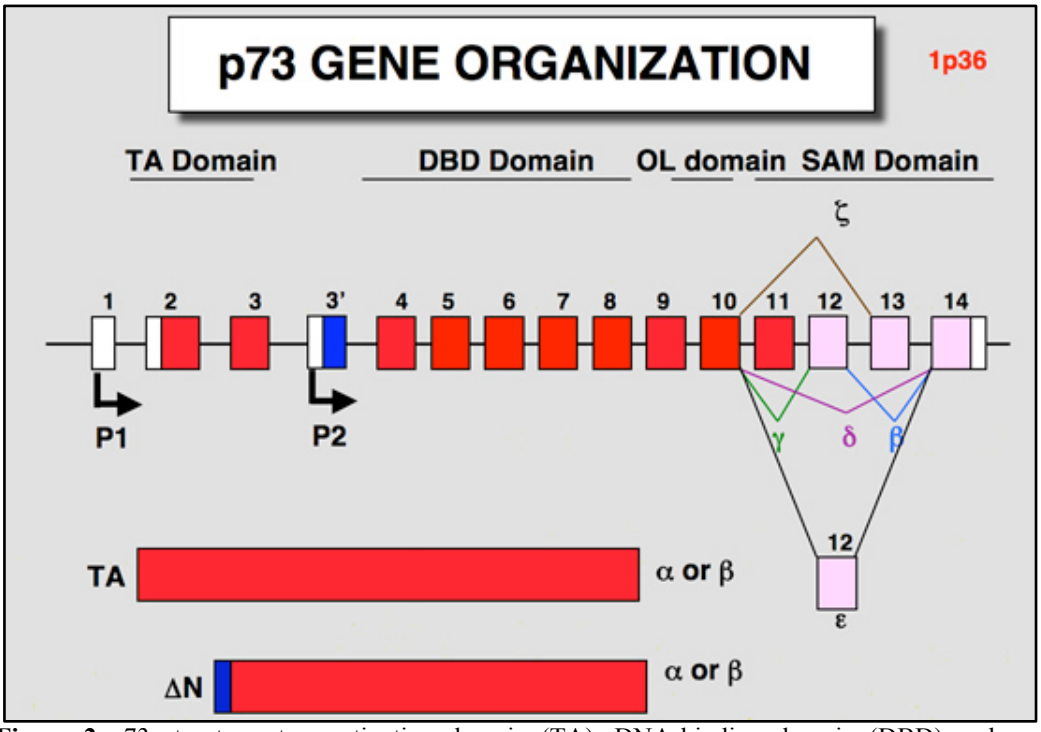


Figure 2 p73 structure: transactivation domain (TA), DNA-binding domain (DBD) and oligomerization domain (OL). The α isoforms contain in addition a sterile α motif (SAM) domain and a transactivation inhibitory domain (TID) at their C-termini. Adapted from: http://p53.free.fr/p53_info/p73_p63.html

As a transcription factor, p73 shares structural and functional similarities with p53 [Yang et al., 2000]. p53 and p73 are reported to share significant homology in the three domains: trans-activation domain (TA), DNA binding domain DBD) and tetramerization domain (TD), also known as oligomerization domain (OL). The c-termini of the α isoforms also contains sterile α motif (SAM) domain (Figure 2) [Ozaki et al., 2005]. As a consequence, p73 can bind to p53 response elements and activate p53 target downstream genes. p73, like p53, is maintained at low

levels in cells, and activation of p73 is regulated mostly at the post-translational level, including ubiquitination, phosphorylation, prolyl-isomerization, acetylation and recruitment into PML-nuclear body [Jost et al. 1997]. Unlike p53, p73 contains two distinct promoters, which form two proteins of contradicting effects: TAp73 exhibits pro-apoptotic effects; whereas, ΔNp73 exhibits anti-apoptotic effects. Modulation in relative levels of these two proteins has been implicated in prognosis in multiple cancers.

p53 induces apoptosis via regulating p53-dependent apoptotic-related genes; such as Fas, DR5, Bax, Noxa and Bcl-2 [Yakovlev et al., 2004; Mendoza-Rodríguez et al., 2001]. Whereas, p73 has been reported to induce apoptosis via three well established mechanisms: 1) TAp73 can activate ER stress via transactivation of Scotin, a transmembrane ER resident protein [Terrinoni et al., 2004]. It has been observed that upregulation of Scotin induces caspase dependent apoptosis in p53 null cancer cells, while blocking Scotin results in reduction of p53 dependent apoptosis, 2) TAp73 can directly transactivate both PUMA and Bax promoters, resulting in mitochondrial dependent apoptosis [Melino et al., 2004], 3) TAp73 can bind to the promoter of Fas (CD 95), which contains a p53 responsive element, thereby activating the apoptotic pathway [Zhu et al., 1998]. Thus, p73 can induce apoptosis via multiple pathways depending on the cell origin type, cancer type, type of DNA damage and type of chemotherapeutic agent used [Ramadan et al., 2005]. Therefore, a complete understanding of p73

mediated pathways is required for development of effective cancer therapies.

1.2.3. Inhibiting growth factor receptors and their down-stream substrates

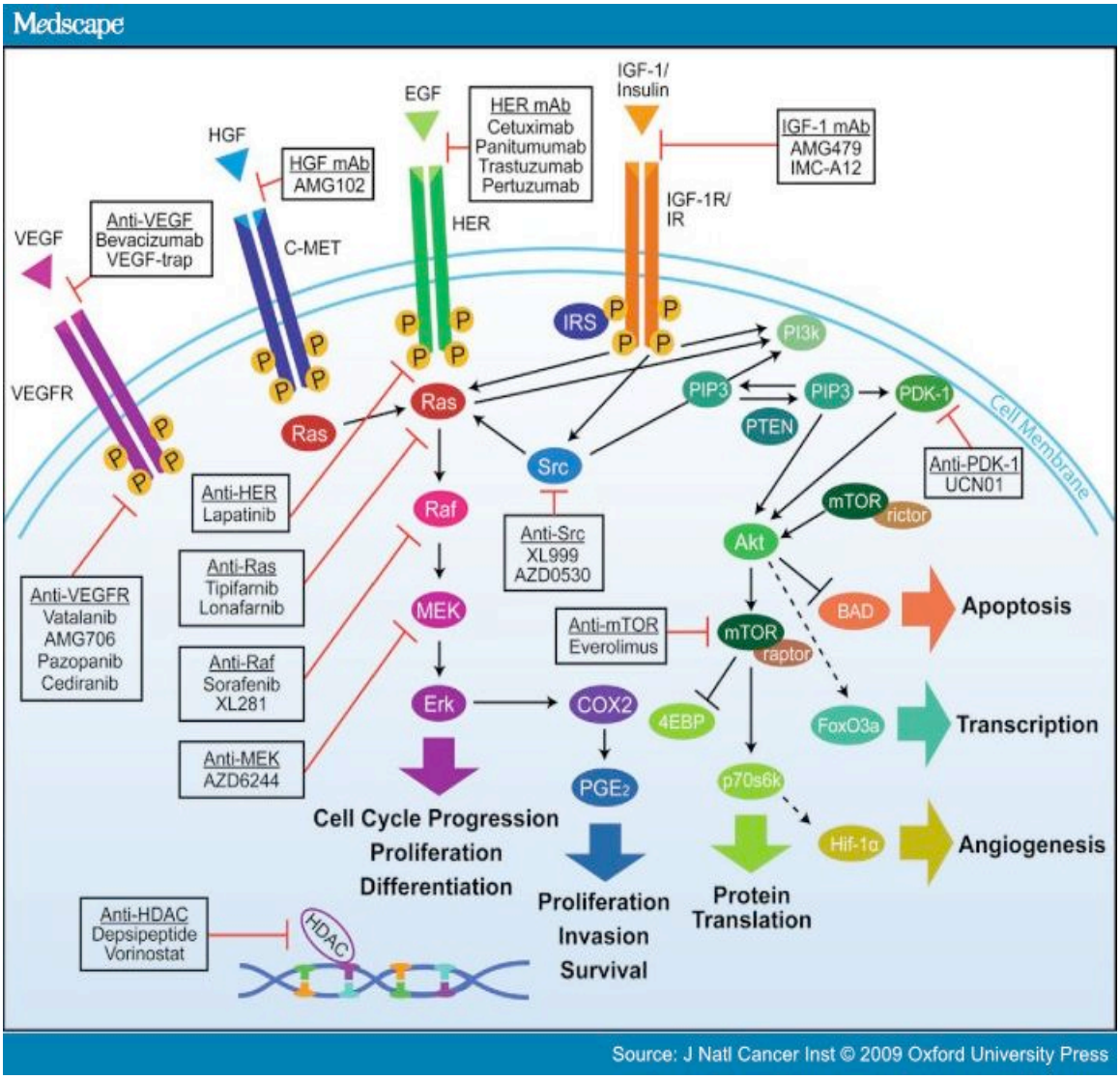


Figure 3 A schematic of inhibiting growth factor signaling pathway by various clinically relevant drugs. Adapted from: Journal of National Cancer Institute

A schematic of growth factor signaling pathways are shown in Figure 3. The epidermal growth factor receptor family consists of: Her-1 (EGFR), Her-2/neu, Her-3 and Her-4. Binding of the specific ligands to Her-1, Her-3 and Her-4 activates these these receptors. Her-2 is referred to as an "orphan receptor" since it doesn't bind with any ligand directly, rather dimerizes with ligand bound Her-1, Her-3 or Her-4. Receptor dimerization activates intracellular signaling pathways, leading to cell growth, proliferation, and survival [Schlessinger et al., 2000]. Her-2 gene is reported to be amplified in 20% of breast cancers [Harari et al., 2000]. The overexpression of Her-2 protein causes increased signal pathway activation, which contributes to the uncontrolled growth and survival of these cancers. Tumors overexpressing Her-2 are considered to be more aggressive and patients with these tumors have a poorer prognosis and reduced chance of survival. Recently, there have been significant developments in strategies to inhibit Her-2 signaling, including small molecule inhibitors (Lapatinib) as well as monoclonal antibodies (Herceptin) [Tevaarwerk et al., 2009; Mannocci et al., 2010].

The insulin like growth factor (IGF) signaling pathway plays a major role in normal breast development, and breast cell remodeling during pregnancy and lactation. The binding of IGF-1 to insulin like growth factor 1 receptor (IGF1R) activates downstream intracellular signaling leading to cell growth, proliferation and differentiation [Werner et al., 2009]. Preclinical studies have reported that dysregulation of IGF signaling pathway promotes transformation, survival,

growth and metastasis of breast cancer cells. Epidemiologic studies have reported high circulating levels if IGF-1 in premenopausal women with breast cancer and Immunohistochemical staining has confirmed overexpression of IGF1R in nearly half of all breast cancers [Frasca et al., 2008]. There are reports confirming crosstalk of IGF1R with epidermal growth factor receptor family (Her-1, Her-2). Currently, there are many therapies to inhibit the IGF/IGFR signaling pathway. One of the strategies is to reduce circulating IGF1 levels using hormone inhibitors [Weroha et al., 2008]. Another strategy is to interfere with the binding of IGF-1 to IGF1R. Several monoclonal antibodies directed against IGF1R (Cixutumumab, Figitumumab) are being developed. Small molecule inhibitors of IGF1R are currently in use [Hewish et al., 2009].

1.2.4. Inhibiting Akt/ERK/mTOR pathway

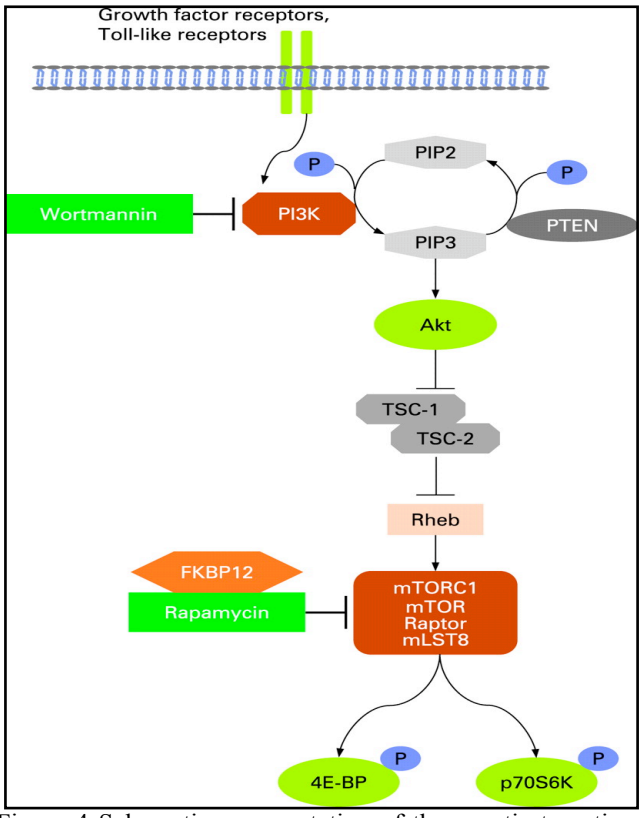


Figure 4 Schematic representation of therapeutic targeting of pro-survival mediators PI3K, Akt, ERK and mTOR. Adapted from: http://ard.bmj.com/content/67/Suppl_3/iii70.abstract

The PI3K, AKT, ERK, and mTOR pro-survival mediators (depicted in Figure 4) are important therapeutic targets since they are constitutively activated in many cancers and contribute to cancer progression by promoting cellular proliferation and inhibiting cell death signaling pathways [McCubrey et al.,

2007]. PI3K is activated at the cell membrane by tyrosine kinase growth factor receptors [Schlessinger et al., 2000]. PI3K promotes cancer cell survival by activation of downstream mediators AKT and Ras, the latter leading to ERK activation. AKT controls key cellular processes, including apoptosis, cell cycle progression, transcription, and translation [Chang et al., 2003]. A major downstream substrate of AKT is the serine/threonine kinase mTOR. AKT can directly phosphorylate mTOR and activate it, which then signals to its downstream effectors S6 kinase/ribosomal protein S6 (p70S6K) and the eIF4E binding protein (*p4E-BP1*) to control transcription and translation, which selectively regulates multiple proteins that control cell cycle and apoptosis [Sun et al., 2005]. ERK exerts its anti-apoptotic effects by phosphorylating and inactivating Bad.

As with most intracellular signaling cascades, cross-talk and negative and positive feedback loops complicate final signaling outcomes. For example, the mTOR substrate p70S6K can ultimately diminish pro-survival signaling via PI3K/AKT by catalyzing an inhibitory phosphorylation site on insulin receptor substrate-1, an upstream mediator of PI3K [Wan et al., 2007]. Likewise, ERK can diminish pro-survival signaling by PI3K/AKT via p70S6K [Jiang et al., 2009]. Therefore, although ERK and mTOR showed potential as anticancer targets, inhibitors of ERK or mTOR alone are limited in clinical application due to the mitigation of these negative feedback loops essential for controlling AKT activity

[Sun et al., 2005]. Thus, this more in depth understanding of signaling pathways suggests that ERK or mTOR inhibitors need to be combined with agents that can circumvent the loss of negative feedback controls on AKT and/or effectively block AKT activity.

1.3 Apoptosis

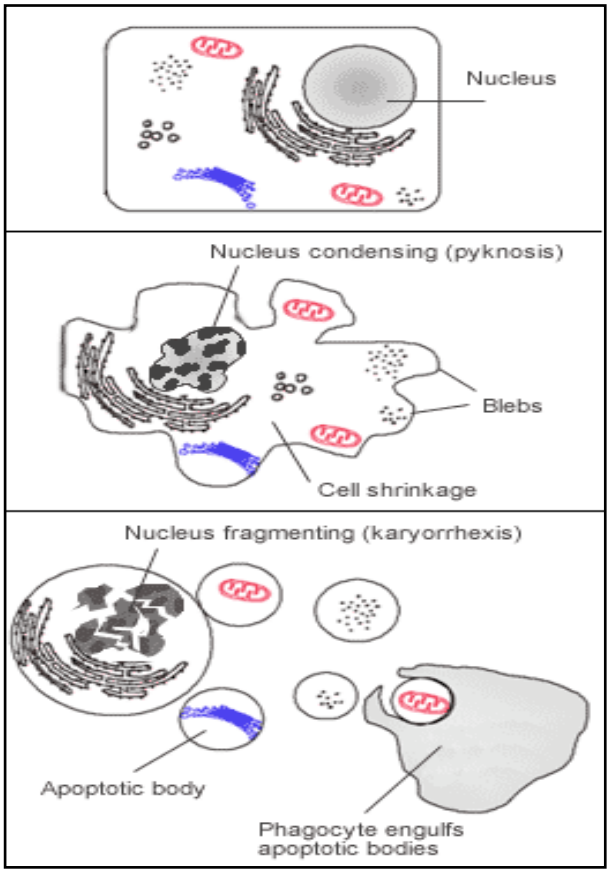


Figure 5 Schematic representation of cell undergoing apoptosis.
Adapted from: http://upload.wikimedia.org/wikipedia/commons/8/86/Apoptosis.png

Apoptosis defined as programmed cell death (PCD), occurs during embryogenesis and maintains homeostatis. Cells undergoing apoptosis have the following distinct morphological characteristics: membrane blebbing, cell shrinkage, nuclear fragmentation chromosomal and DNA fragmentation and formation of apoptotic bodies, which are eventually engulfed by phagocytes *in vivo* (Figure 5) [Steller et al., 1995].

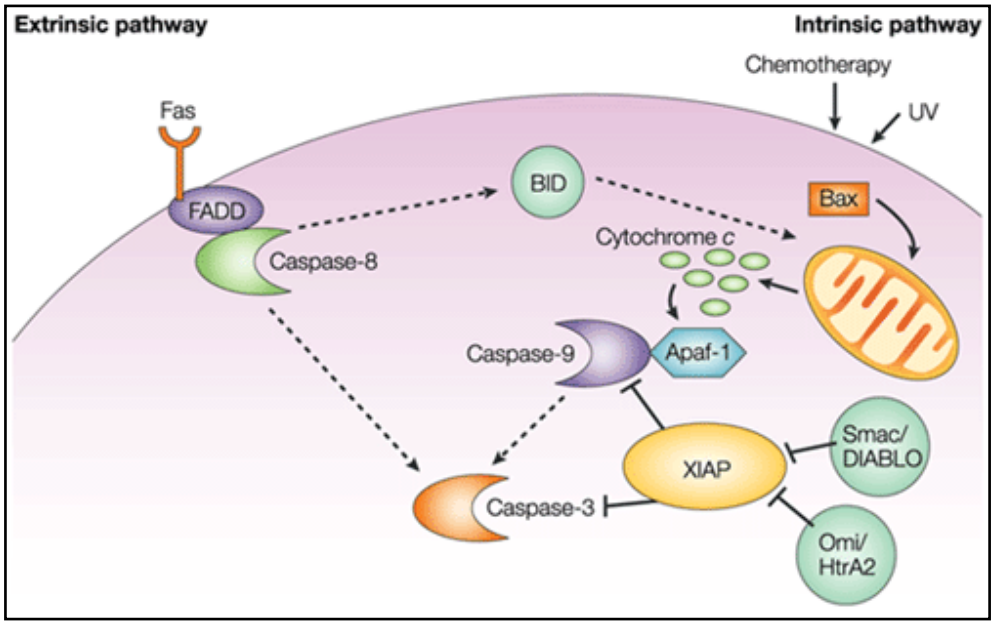


Figure 6 Schematic representation of extrinsic and intrinsic apoptosis.
Adapted from: http://www.nature.com/embor/journal/v5/n7/fig_tab/7400191_f1.html

Signaling pathways must be activated either intrinsically or extrinsically for a cell to under apoptosis (Figure 6). The intrinsic pathway that can be activated by different stresses including hypoxia, chemotherapy, UV radiation, DNA damage, is a mitochondrial dependent process. Mitochondrial membrane permeability (MMP) is regulated by Bcl-2 family members. Loss of MMP triggers release of cytochrome c, which then associates with Apaf-1 and pro-caspase-9 to form the apoptosome, which then activates initiator caspase 9 and effector caspase-3, ultimately leading to apoptosis [Cain et al., 2000]. The extrinsic pathway can be activated by interaction of surface death receptors (DR) with the cytokines of tumor necrosis factor (TNF) family including TNF, TNF-

related apoptosis inducing ligand (TRAIL), and Fas ligand [Wang et al., 2003]. This binding induces homotrimerization of the receptor and recruitment of pro-caspase-8 and FADD to form disc inducing signaling complex (DISC). DISC then cleaves pro-caspase-8 to active caspase-8 and other effector caspases consequently [Peter et al., 2003]. These two pathways are not mutually exclusive but there is crosstalk between them, which is mediated by pro-apoptotic Bcl-2 homology 3 (BH3), Bid, which is cleaved by extrinsic pathway activated caspase-8. Truncated Bid (tBid) then translocates to mitochondria membrane and inhibits anti-apoptotic Bcl-2, allowing Bax to facilitate release of cytochrome c, resulting in the formation of apoptosome and subsequently activation of the intrinsic pathway [Fulda et al., 2006].

1.4 Vitamin E

1.4.1 Structure and functions of vitamin E

Vitamin E was discovered by Evans and Bishop in 1922 as a micronutrient essential for rat reproduction. Vitamin E refers to a group of essential, fat-soluble antioxidants, including eight different naturally occurring forms called α-, β-, γ-, δ-tocopherols and tocotrienols, as well as synthetic vitamin E (*all-rac*-α-tocopherol) [Kline et al., 2004]. All forms of vitamin E contain a chroman head and phytyl tail. Tocopherols and tocotrienols differ in number of double bonds on the phytyl tail as well as the position of methyl groups on the chroman head [Mustacich et al., 2007]. Phytyl tails of tocopherols are saturated; whereas, phytyl tails are unsaturated in tocotrienols. Tocopherols consists of three asymmetric carbons at the 2^{nd} position on the chroman head and at the 4^{th} and 8^{th} positions on the phytyl tail, either right handed or left handed designated as R and S respectively. All the three positions in the naturally occurring vitamin E forms have right-handed carbons and thus referred as RRR-α-tocopherol (α-T), the most preferentially retained form of vitamin E in humans [Kline et al., 2004].

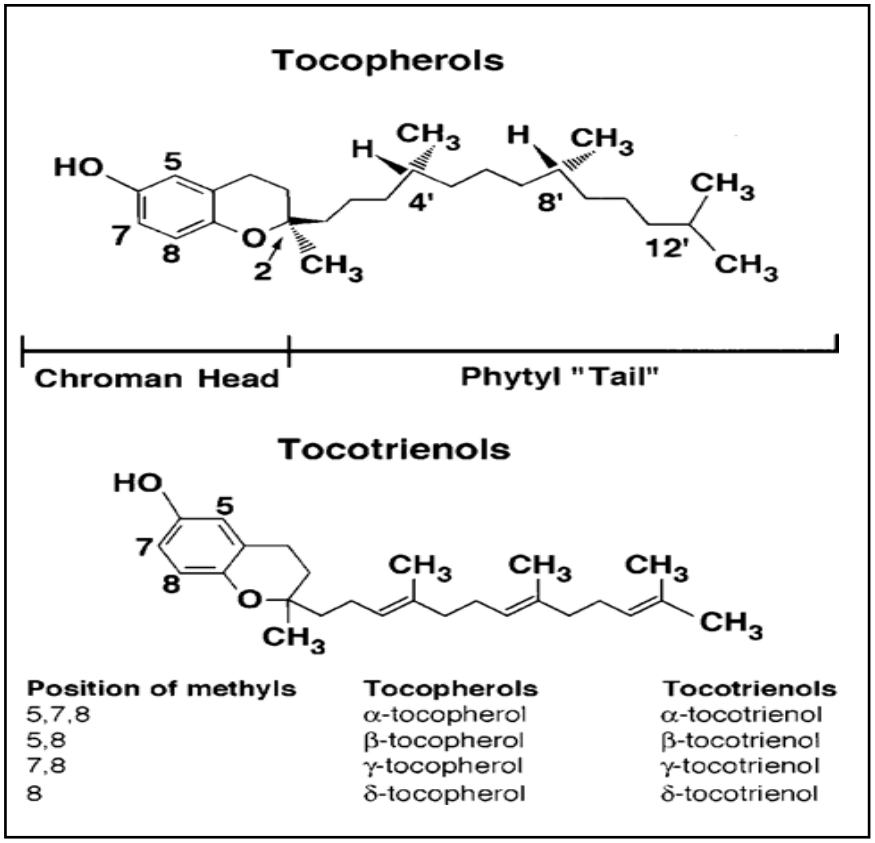


Figure 7 Structures of naturally occurring tocopherols and tocotrienols. Tocopherols and tocotrienols differ in that tocopherols have a saturated side chain referred to as a phytyl tail and tocotrienols have an unsaturated (isoprene) side chain. The four different forms of tocopherols and tocotrienols differ by the presence and position of methyl (CH3) groups on the aromatic ring of the molecule. For example, alpha forms have three methyl groups at the 5, 7, and 8 positions while delta forms have only a single methyl group at the 8 position. Tocopherols have three chiral carbon-centers at positions C-2, C-4′, and C-8′. Tocotrienols have one chiral carbon at position C-2. All natural forms of vitamin E are R-stereoisomers. Adapted from (Kline, Lawson et al. 2003).

Plants are major sources of naturally occurring vitamin E, RRR-α-tocopherol is found abundantly in sunflower oil [~6 mg/Tb], cottonseed oil [~5 mg/Tb], and safflower oil [~5 mg/Tb], whereas tocotrienols are highly

concentrated in wheat germ oil [~3 mg/Tb] and palm oil [~6 mg/Tb] [Wikipedia]. Vitamin E supplements that are commercially available can contain either naturally isolated α-T or synthetic forms or derivatives of αT.

According to the clinical reports, different vitamin E forms are known to reduce risks of chronic diseases such as neurodegenerative diseases, cardiovascular diseases and artherosclerotic progression. αT is a fat soluble anti-oxidant and some of its benefits include being anti-inflammatory, anti-thrombotic and anti-cancer [Kline et al., 2004].

1.4.2. Synthetic derivatives of vitamin E

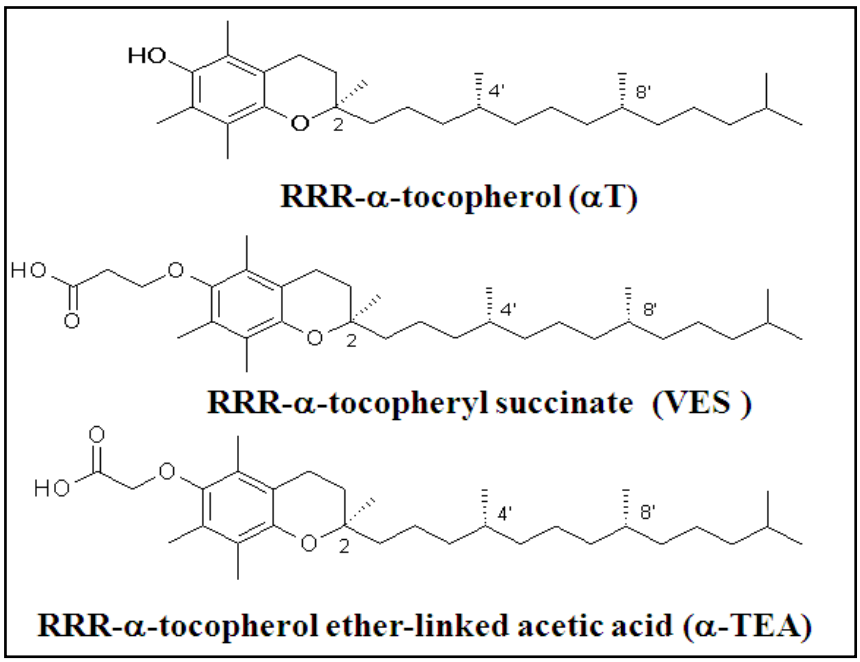


Figure 8 Structures of αT, VES and α-TEA.
RRR-a-tocopheryl succinate (VES), an ester-linked succinic acid analog of RRR-a-tocopherol and [(2,5,7,8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl) chroman-6-yloxyacetic acid], an ether-linked acetic acid analogue of RRR-a-tocopherol (a-TEA). Adapted from (Kline, Lawson et al. 2003).

The hydroxyl moiety at the carbon 6 position is prone to oxidation when exposed to air and since this hydroxyl is critical for the antioxidant effect of vitamin E, it must be removed to maintain potency. Thus two synthetic forms that are resistant to oxidation were developed: R,R,R- α–tocopheryl succinate, α–TOS (VES), in which succinic acid is coupled to the carbon 6 position on the chroman head via an ester bond. The other is acetate derivative referred as α–tocopheryl acetate

(α–T-Ac) [59]. Both these pro-vitamin E forms are converted to active free α–T by pancreatic enzymes after the oral intake. Suprisingly, only the succinate derivative (α–TOS) showed potential for apoptogenic activity *in vitro* and anti-tumor and anti-metastatic effects *in* vivo in comparison to α–tocopheryl acetate (α–T-Ac) [60]. However, the clinical use of α–TOS is obstructed due to the presence of intestinal esterases, which hydrolyze the succinate moiety, yielding α–T and succinic acid, neither of which exhibits anti cancer functions [61]. To overcome the hydrolytic inactivation by esterases, our lab developed a non hydrolysable ether linked acetate moiety at the carbon 6 position, referred as 2,5,7,8-tetramethyl-2R-(4R, 8R-12-trimethyltridecyl)chroman-6- yloxyacetic acid (α– tocopheryloxyacetic acid or α–TEA) [62]. Unlike α-TOS, the ether linkage is nonhydrolyzable and the molecule remains stable *in vivo*. For this reason, α-TEA is not known to have antioxidant properties [63]. However, α-TEA exhibits pleiotropic anti-tumor effects specifically to cancer cells such as induction of apoptosis and differentiation, inhibition of cell survival, proliferation and metastasis. It has been also observed that α-TEA can synergize with other chemotherapeutic agents to induce cancer cells to undergo apoptotic cell death.

1.4.3. Anti-cancer mechanisms mediated by α-TEA

α-TEA was developed by our lab in search of clinically relevant vitamin E based chemotherapeutic agents. α-TEA induces apoptosis in human breast cancer cells (MCF-7, MDA-MB-435, MDA-MB-231, MDA-MB-468, BT-20, HCC-1954, MDA-MB-453), human prostate cancer cells (LNCaP, PC-3, DU-145), endometrial cells (RL-952), lung (A-549), colon (HT-29, DLD-1) and lymphoid (Raji, Ramos, Jurkat) cells [Wang et al., 2008; Jia et al., 2008; Shun et al., 2004; Tiwary et al., 2010]. Interestingly, α-TEA does not induce apoptosis in normal human prostate epithelial cells (PrEC) or normal human mammary epithelial cells (HMEC), making it an ideal chemotherapeutic agent [Anderson et al., 2004].

α-TEA has also been shown to be effective at reducing tumor burden and metastasis in both xenograft models with transplanted human breast cancer cells (MDA-MB-435-GFP), human prostate cancer cells (PC-3-GFP) or cisplatin-resistant ovarian cancer cells (A2780-CP70-GFP) as well as syngeneic mouse mammary tumor models (66cl-4-GFP and 4T1) [Wang et al., 2008; Jia et al., 2008; Lawson et al., 2003; Latimer et al., 2009]. Also, combinations of α-TEA with other chemotherapeutic agents such as cyclooxygenase-2 inhibitor celecoxib, 9-nitro-camptothecin, paclitaxel and cisplatin were more effective in reducing tumor burdens and inhibiting metastasis than single agent treatment [Latimer et al., 2009; Zhang et al., 2004; Anderson et al., 2004].

α-TEA is water insoluble crystalline chemical and can be delivered by

multiple ways. α-TEA was less effective when administered by oral gavage using peanut oil (87.5%) with ethanol (12.5%) as a solvent [Lawson et al., 2003]. Whereas, when α-TEA was formulated in liposomes and delivered via aerosol or oral gavage, both delivery routes were effective in suppressing tumor growth and metastasis *in vivo* [Wang et al., 2008; Lawson et al., 2003]. Another effective route for administering α-TEA is by incorporating it into diet.

Since, α-TEA has been proven to be effective in significantly reducing tumor burden and metastasis *in vivo* and inducing apoptosis and inhibiting cell proliferation in a variety of cancer cells *in vitro*, intensive efforts have been employed to study the mechanisms of action and possible targets of α-TEA. α-TEA induces cancer cells to undergo apoptosis by activating pro-apoptotic pathway mediators and simultaneously inhibiting pro-survival mediators. Studies conducted in our lab have shown that α-TEA activates the pro-apoptotic pathway via enhancing Fas signaling, a cell surface death receptor, leading to caspase 8 activation which causes translocation of tBid to mitochondria, release of cytochrome c, formation of apoptosome, activation of caspase-9 and effector caspases as well as PARP, ultimately causing apoptosis [Wang et al., 2008; Jia et al., 2008]. Previous studies also implicated activation of c-Jun N-terminal kinase (JNK) in α-TEA induced apoptosis in both breast and prostate cancer cells. It was observed that binding of Fas ligand to the Fas receptor activates two downstream signaling pathways: 1) classical death receptor, leading to activation of caspase 8,

Bid, caspase 9, effector caspases, causing apoptosis and 2) by activation of JNK via Daxx, a death domain associated protein. JNK upon activation activates downstream transcription factor, c-Jun, which binds directly to the promoter region of Fas and FasL, thereby enhancing gene expression level of both Fas and FasL [Jia et al., 2008, Yu et al., 2010]. Another study reported that breast cancer cells when treated with α-TEA activated JNK, which in turn regulated p73, a member of p53 family (Figure 2). Furthermore, data suggested that upregulation of p73 caused upregulation of NOXA. cDNA microarray analyses of α-TEA treated human breast cancer cells revealed that over 400 genes were regulated [Wang et al., 2008].

Additionally, it has been observed that α-TEA inhibits pro-survival pathway by downregulating the pleiotropic anti-apoptotic factor Akt. Further studies revealed that α-TEA also downregulates EGFR (namely, Her-1, Her-2 and Her-3) as well as FLICE-like inhibitory protein (FLIP) and survivin protein levels [Shun et al., 2010]. As discussed previously, overexpression of pro-survival pathway has been correlated with poor prognosis and has also been thought to cause drug resistance. α-TEA can enhance pro-apoptotic pathways and inhibit pro-survival pathways, making it a unique agent for both preventive and therapeutic purposes.

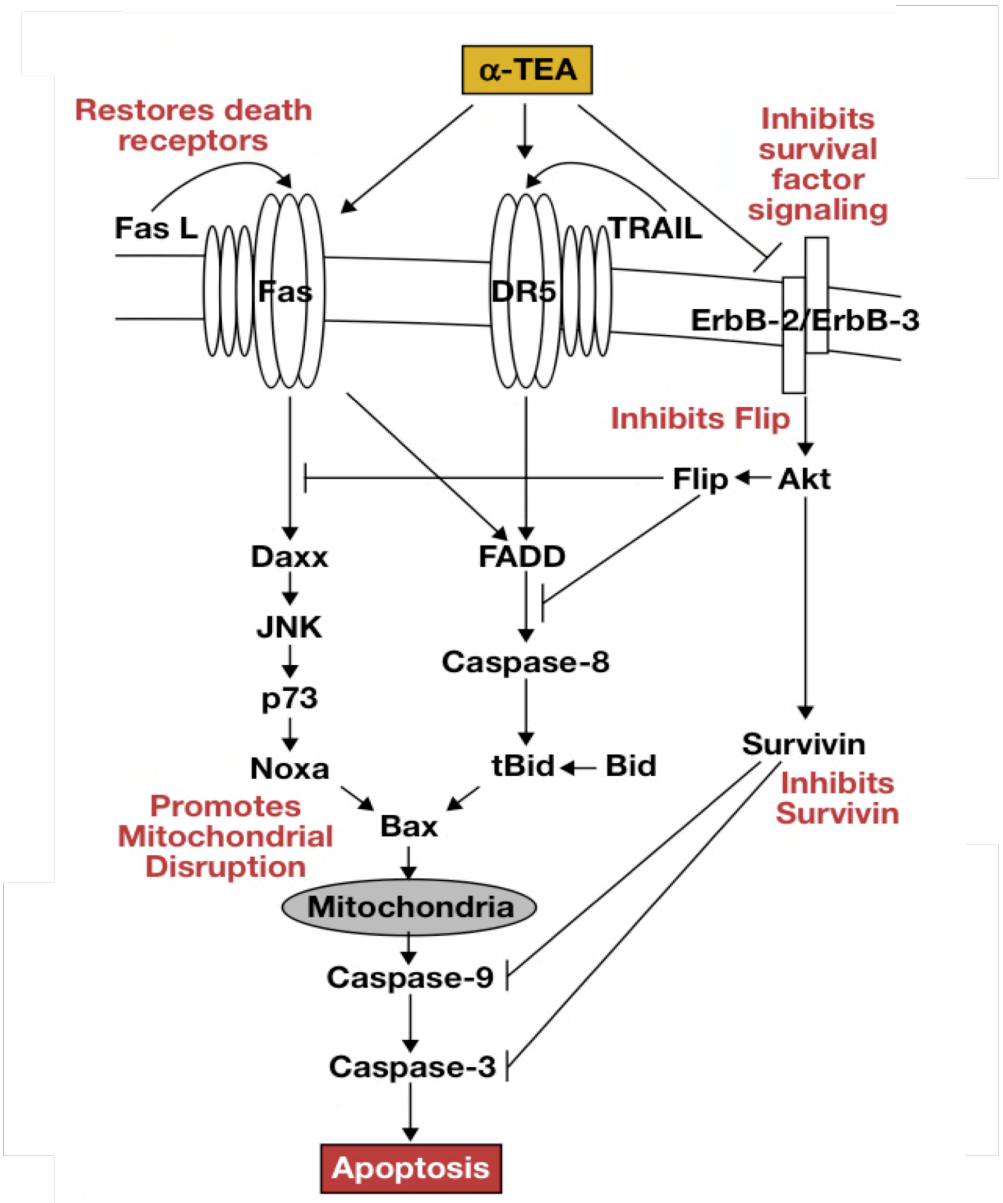


Figure 9 Schematic representation of mechanism of action of α-TEA. α-TEA modifies aberrant signaling pathways in a wide range of major cancers by restoration of endogenous death signaling receptors, down-regulation of inhibitors of apoptosis, reversal of mitochondria survival mode, and activation of death signaling caspases, resulting in cancer cell death.

1.4.4. Endoplasmic Reticulum (ER) stress and apoptosis

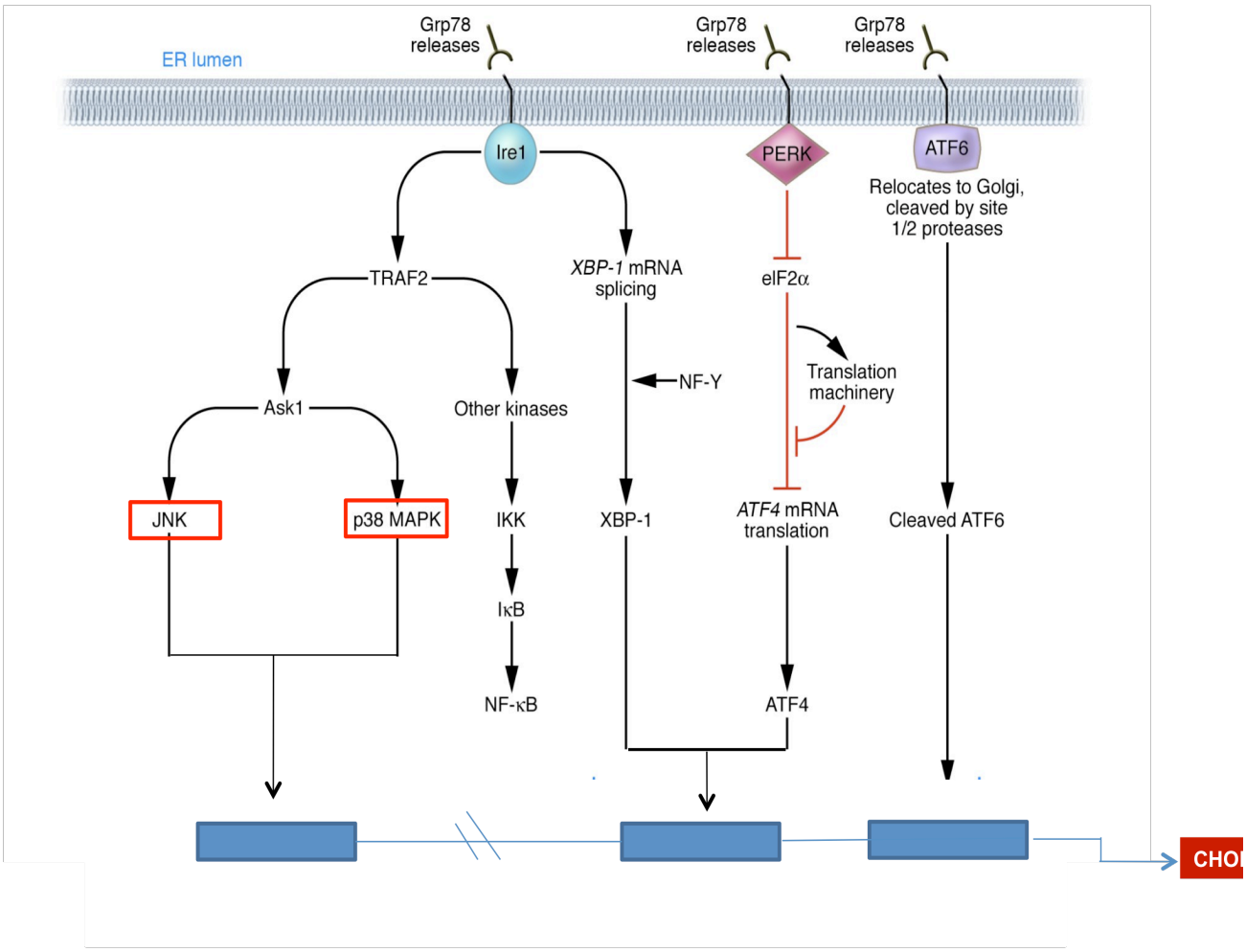


Figure 10 Schematic representation of ER stress. Upon accumulation of unfolded proteins, GRP78 is activated, thereby releasing IRE1, PERK and ATF-6. Once PERK is activated, it phosphorylates eIF2α thereby inducing global repression of translation. IRE-1 upon activation, phosphorylates ASK-1 and also splices XBP-1. ATF-6 translocates from ER to Golgi and finally into nucleus. ATF-4, ATF-6 and XBP-1 directly bind to cis acting elements of CHOP and activates it. Modified from Xu C et al. J Clin Invest. 2005 ,115(10):2656-64

In eukaryotes, endoplasmic reticulum (ER) is an organelle in which secretory and transmembrane proteins are synthesized, folded or modified. Molecular chaperones and protein foldases are required for proper protein folding. An unfolded protein is characterized by large fraction of hydrophobic amino acids side chains displayed on its surface than in its native conformation. These unfolded proteins can aggregate and interfere with normal protein folding. Perturbations in the efficiency of protein folding can lead to the accumulation of these misfolded or unfolded protein can cause a cell to deviate from homeostatis, threatening its survival and leading to ER stress [Schröder et al., 2005]. Several physiological changes such as ischemic insults, depletion of calcium, hypoxia, glucose starvation as well as expression of mutated proteins can cause ER stress leading to unfolded protein response (UPR). In order to cope with ER stress, cells initially attenuate translation to reduce the load of ER. In the next phase, genes are transcriptionally induced for long term adaptation to ER stress. ER chaperones are induced to refold the misfolded protein and if unsuccessful, ERAD components are induced to eliminate these misfolded proteins. Stressed cells also induce NF-κB to elicit immune response and anti-apoptotic pathways. However, if stress conditions persist, apoptotic pathways are activated [Shen et al., 2004].

Three functionally different ER stress sensors reside on the ER membrane: 1) type I transmembrane protein kinase endoribonuclease, inositol-requiring enzyme 1 (IRE-1), 2) pancreatic ER kinase like (PERK) and 3) basic leucine

zipper activating transcription factor 6 (ATF-6). In resting cells, all these ER stress sensors are maintained in an inactive state through binding with ER chaperone, BiP (also known as HSPA5 and GRP78). However, under ER stress conditions, BiP is sequestered through binding to unfolded or misfolded proteins, thereby releasing and consequently activating the ER stress sensors [Xu et al., 2005].

The release of Bip causes activation homodimerization and trans-autophosphorylation of PERK, thereby activating PERK. Once activated, PERK phosphorylates and thereby inactivates the translation initiation factor eIF2α, which is responsible for mRNA translation initiation, leading to attenuation of protein translation. However, ATF-4 mRNA is preferentially translated by phosphorylated eIF2α. ATF-4 is a transcription factor known to activate transcription of UPR target genes such as Bip and GRP94 [Shi et al., 1998].

Bip also releases ATF-6, allowing ATF-6 to translocate to Golgi apparatus, where it is cleaved and thereby activated by proteases S1P and S2P. ATF-6 regulates transcription of UPR target genes such as X-box binding protein 1 (XBP-1) and Bip, which facilitate elimination of misfolded protein. Release of IRE-1 from Bip, dimerizes IRE-1 leading to autotransphosphorylation, thereby activating endoribonuclease activity of IRE-1. IRE-1 splices XBP-1 by removing a 26 base intron from XBP-1 mRNA [Rao et al., 2004]. Spliced XBP-1 encodes a potent transcription factor that when translocated to nucleus, activates expression

of UPR target genes. IRE-1 activation also leads to recruitment of two apoptotic factors: adaptor protein, tumor necrosis factor receptor associated factor 2 (TRAF-2) and apoptosis signal regulating kinase 1 (ASK-1). ASK-1 when activated by ER stress, activates its downstream targets: JNK and p38 MAPK. Activation of JNK ultimately leads to apoptosis of the cell via inhibiting pro-survival Bcl-2 protein [Xu et al., 2005].

The transcription factors namely, ATF-4, XBP-1 and ATF-6 once activated, bind to *cis* acting elements in the promoter region of CHOP (also known as GADD 153), thereby upregulating the transcriptional activity of CHOP. In addition to transcriptional regulation, CHOP expression levels can be modulated post-transciptionally by mRNA stability [Zinszner et al., 1998]. Under ER stress conditions, p38 MAPK phosphorylates CHOP at Ser 78 and Ser 81, enhancing both the transcriptional and apoptotic effect of CHOP. Overexpression or microinjection of CHOP has been shown to induce apoptosis and/or cell cycle arrest, whereas attenuation of CHOP results in marked inhibition of ER stress induced apoptosis. Overexpression of CHOP has been associated with downregulation of anti-apoptotic protein Bcl-2, however whether CHOP binds directly to the promoter region of Bcl-2 is still unknown [Thomenius et al., 2003]. One of the well known downstream target of CHOP is mammalian homolog of yeast ER oxidase (ERO1α), which once activated promotes oxidizing conditions in stressed cells via accumulation of reactive oxygen (ROS), leading to cell death.

GADD34 is another well known target of CHOP. CHOP directly activates GADD34, which promotes protein biosynthesis by reversing the translation repression via dephosphorylating Ser 51 of the α-subunit of translation initiation factor 2 (eIF2α) in stressed cells [86]. Recently, it has been observed that CHOP also mediates upregulation of death receptor 5 (DR5), leading to apoptosis [Tiwary et al., 2010]. Furthermore, it was seen that overexpression of CHOP causes Bax translocation from cytosol to mitochondria, causing mitochondrial dependent apoptosis.

Chapter 2. Role of Endoplasmic Reticulum Stress in α-TEA mediated TRAIL/DR5 Death Receptor Dependent Apoptosis

2.1. Introduction

Targeting cell surface death receptors, especially tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) binding receptors, holds promise for cancer treatment [Ashkenazi, 2008; Kruyt, 2008]. TRAIL selectively induces apoptosis in a wide variety of cancer cells with little or no toxicity towards normal cells [Ashkenazi, 2008; Kruyt, 2008]. Thus, agents that can enhance TRAIL death receptor (TRAIL-R/DR4 or TRAIL-R2/DR5) signaling or sensitize TRAIL resistant cells to TRAIL induced apoptosis are of interest [Kruyt, 2008; Seok-Hyun et al., 2008]. TRAIL/DR4/DR5 apoptotic signaling includes: interaction of TRAIL with DR4 or DR5, receptor clustering, recruitment of the adaptor molecule FADD, and activation of initiator caspases-8 or -10, leading to cleavage of downstream effector caspases (mitochondrial-independent apoptosis) or cleavage of Bid, a pro-apoptotic Bcl-2 family member, leading to mitochondrial-dependent apoptosis [Jin et al., 2005].

Evading apoptosis is a hallmark of cancer [Hanahan et al., 2000]. One way tumor cells can escape death signals is by expression of anti-apoptotic pro-survival proteins [Spencer et al., 2009]. Therefore, targeting anti-apoptotic proteins also holds promise for killing cancer cells and sensitizing them to

different therapeutics [Tuma, 2009]. c-FLIP (cellular FADD-like IL-1α-converting enzyme inhibitory protein), is a death effector domain containing protein that regulates extrinsic death receptor signaling from the tumor necrosis factor-α (TNF-α) family of cell surface death receptors, including DR4/DR5, Fas (CD95/APO-1), and TNFR [Safa et al., 2008]. c-FLIP is a catalytically inactive caspase-8/10 homolog and typically functions as a caspase-8 inhibitor resulting in chemotherapeutic drug resistance [Safa et al., 2008].

α-TEA[2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid], called RRR-α-tocopherol ether-linked acetic acid analog or RRR-α-tocopheryloxyacetic acid is a nonhydrolyzable ether analog of RRR-α-tocopherol [Lawson et al., 2003]. α-TEA has been shown to be a potent pro-apoptotic agent both in *vitro* and *in vivo* in breast, prostate and ovarian cancer cells [Anderson et al., 2004; Lawson et al., 2004; Hahn et al., 2006; Jia et al., 2008; Yu et al., 2006; Kline et al., 2007]. Recently, α-TEA has been shown to delay tumor onset and inhibit the progression and metastatic spread in a clinically relevant model of spontaneous breast cancer, further highlighting the translational potential of this anticancer agent [Hahn et al., 2009]. Mechanisms involved in α-TEA induced apoptosis include: activation of JNK/c-Jun, p73/NOXA and Fas, as well as suppression of c-FLIP-L, survivin and phospho-Akt (pAkt), leading to death receptor mediated caspase-8 activation and mitochondria dependent

apoptosis [Shun et al., 2004; Yu et al., 2006; Jia et al., 2008; Yu et al., 2009; Wang et al., 2008; Shun et al., 2010].

These data are the first to show that α-TEA induces ER stress dependent increases in death mediators JNK/CHOP/DR5 and decreases in survival mediators c-FLIP-L and Bcl-2 in human breast cancer cells. These ER stress mediated events function downstream of α-TEA triggered TRAIL/DR5/caspase-8 signaling, leading to up-regulation of JNK, CHOP and DR5 and downregulation of c-FLIP and Bcl-2.

2.2 Material and Methods

Chemicals: α-TEA was made in house as previously described [Lawson et al., 2004]. ER stress inhibitor salubrinal was purchased from Calbiochem (La Jolla, CA). Caspase-8 inhibitor Z-IETD-FMK was purchased from BioVision (Mountain View, CA).

Cell Culture. MDA-MB-231 estrogen-receptor negative human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). MCF-7 estrogen-responsive human breast cancer cells were originally provided by Dr. Suzanne Fuqua (Baylor College of Medicine, Houston, TX). Both cell lines were cultured in MEM media with 10% FBS. For experiments, FBS was reduced to 2% and cells were allowed to attach overnight before treatments. α-TEA (40 mM) was dissolved in ethanol as stock solution. Equivalent level of ethanol (0.1%) was used as vehicle control (VEH) for α-TEA treatment (40 μM).

Quantification of apoptosis. Apoptosis was quantified by Annexin V-FITC/PI assay following the manufacturer's instructions (Invitrogen).

Western Blot Analyses. Whole cell protein lysates were prepared and western blot analyses were conducted as described previously [Wang et al., 2008]. Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), c-FLIP, CHOP, GRP-78, Bcl-2, total JNK, TRAIL and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA) and Bid (Pharmigen,

Rockville, MD), phospho-eIF-2α (peIF-2α), eIF-2α (eIF-2α), caspase-8, caspase-9, DR5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA).

RT-PCR detection of DR5, Bcl-2 and XBP-1 mRNA expression. Total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect DR5, Bcl-2 and XBP-1mRNA form by reverse transcriptase-polymerase chain reaction (RT-PCR) using the housekeeping gene β-actin as control. 5 μg total RNA was reverse transcribed to cDNA using 1 μl Superscript RTase (250 U, Invitrogen) following the manufacture's instructions. 1 μl cDNA was used per PCR reaction with 15 μl Taq PCR Master Mix Kit (Qiagen Inc) plus 10 μM oligonucleotide primer pairs (Invitrogen). Primer sequences are available upon request.

RNA Interference. A scrambled RNA duplex purchased from Ambion (Austin, TX) that does not target any of the known genes was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of MCF-7 or MDA-MB-231 cells with siRNA to DR5, TRAIL, CHOP, JNK, Itch or control (Ambion, Austin, TX) was performed in 100 mm cell culture dishes at a density of 2 x 10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After one day exposure to transfection conditions, the cells were re-

cultured in 100 mm dish at 2 x 10^6 cells/dish and incubated for one day followed by treatments.

Chromatin immunoprecipiation (ChIP) assay. MDA-MB-231 cells were treated with 40 μM of α-TEA or vehicle (0.1% ethanol) for 15 hours. Protein to DNA cross-linking was conducted by adding 1% formaldehyde to the cell culture medium for 12 min followed by the addition of glycine (0.125M) to stop the cross-linking. Cells were collected for ChIP assay as described [Nelson *et al*., 2006]. The CHOP antibody used for the Western blot analysis was used for immunoprecipitation. Normal mouse IgG_1 purchased from Santa Cruz Biotechnology was used as an isotype control. Polymerase chain reaction (PCR) was conducted using the primers as described [Adbelrahim *et al.*, 2006] to detect CHOP binding sites in the DR5 promoter region.

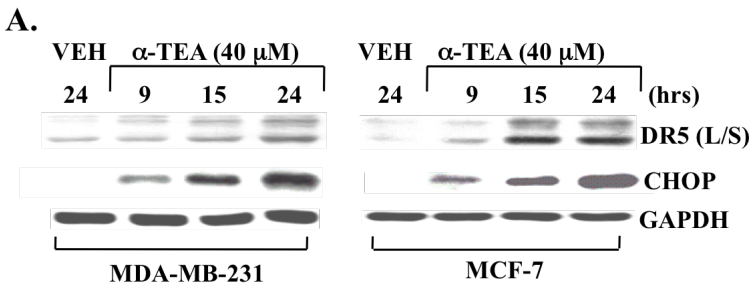
Ectopic Expression of c-FLIP (L). Transient transfection of MCF-7 cells with wildtype His-tagged FLIP expression construct, pcDNA3.1-His-c-FLIP (L), which was kindly provided by Dr. John C. Reed (The Burnham Inst. La Jolla, CA) [Naito et al., 2004] was performed following the procedure published before [Jia et al., 2008]. Transfected cells were cultured overnight before α-TEA treatment.

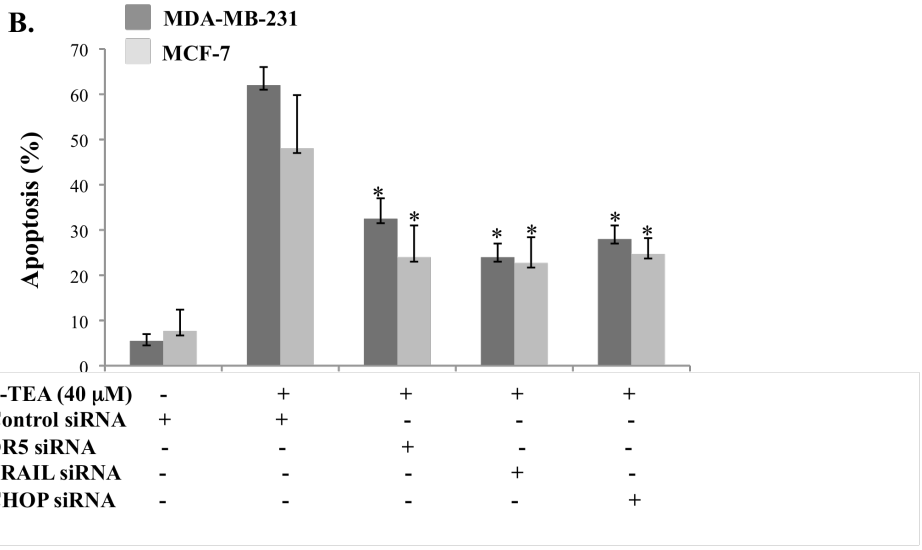
Statistical Analyses. Apoptosis data were analyzed using a two-tailed student *t*-test to determine statistical differences among treatments. Differences were considered statistically significant at $p<0.05$.

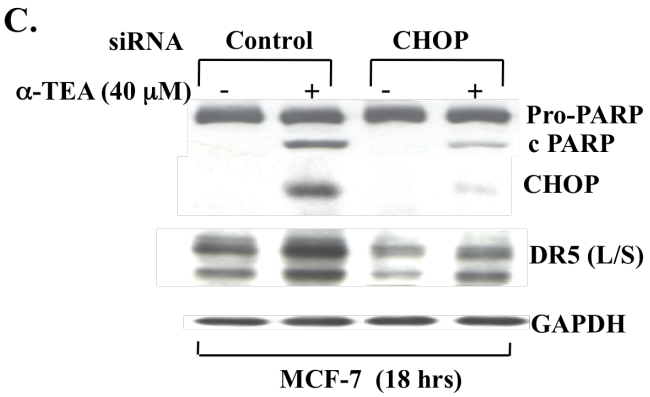
2.3. Results

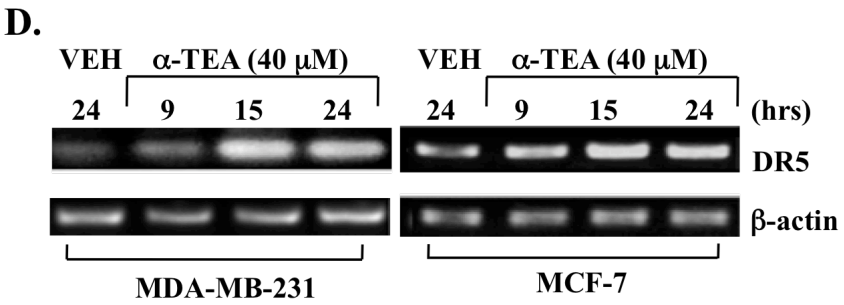
α-TEA induces TRAIL/DR5 and CHOP dependent apoptosis

α-TEA treatment of MDA-MB-231 and MCF-7 breast cancer cells up-regulates DR5 and CHOP protein levels in a time dependent manner (Fig 11 A). siRNA knockdown of TRAIL, DR5 or CHOP significantly reduces the ability of α-TEA to induce apoptosis in both cell lines as detected by FACS analyses of expression of the apoptotic biomarker, Annexin V (Fig 11 B), suggesting that both TRAIL/DR5 and CHOP are involved in α-TEA induced apoptosis. Since CHOP has been shown to act as a positive transcriptional factor for DR5 [Yamaguchi et al., 2004], siRNA to CHOP was used to examine if CHOP regulate α-TEA induced upregulation of DR5. Data show that siRNA to CHOP reduced the ability of α-TEA to increase CHOP and DR5 (L/S) levels, as well as to cleave PARP, a biomarker of apoptosis, in MCF-7 cells (Fig 11C). To further confirm that DR5 is regulated by CHOP in α-TEA induced apoptosis RT-PCR to detect mRNA levels of DR5 and ChIP analyses to assess CHOP binding activity on the DR5 promoter were conducted in MDA-MB-231 cells. Data show that α-TEA induces increased levels of DR5 mRNA (Fig 1D) and increased CHOP binding activity on DR5 promoter (Fig 11E) in comparison to VEH treated control cells. These data indicate that CHOP contributes to α-TEA mediated increases in DR5 protein levels and induction of apoptosis.


A.
VEH
α-TEA (40 μM)
24
9
15
24
(hrs)
DR5 (L/S)
CHOP
GAPDH
MDA-MB-231
MCF-7


B.
MDA-MB-231
MCF-7
Apoptosis (%)
70
60
50
40
30
20
10
0
α-TEA (40 μM) - + + + +
Control siRNA + + - - -
DR5 siRNA - - + - -
TRAIL siRNA - - - + -
CHOP siRNA - - - - +


C.
siRNA
Control
CHOP
α-TEA (40 μM)
-
+
-
+
Pro-PARP
c PARP
CHOP
DR5 (L/S)
GAPDH
MCF-7 (18 hrs)



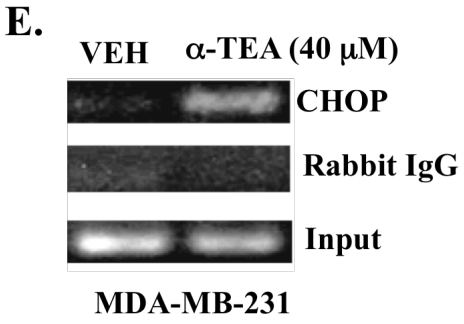


Figure 11. α-TEA induces TRAIL/DR5 and CHOP dependent apoptosis. A. MDA-MB-231 and MCF-7 cells were treated with 40 μM α-TEA for 9, 15, and 24 hrs. Western blot analyses were performed to evaluate DR5 (L/S) and CHOP protein levels using GAPDH as loading control. B. MDA-MB-231 and MCF-7 cells were transiently transfected with siRNAs to DR5, TRAIL or CHOP using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Apoptosis was determined by Annexin V/FACS. C. Samples (from B) were analyzed by western blot for PARP cleavage, CHOP and DR5 (L/S) protein levels using GAPDH as loading control. D. mRNA levels of DR5 were determined by RT-PCR. E. The binding activity of CHOP on DR5 promoter was determined by CHIP assay. Data from A, C, D and E are representative of two or more independent experiments. Data from B are the mean ± S.D. of three independent experiments. * $p<0.05$=significantly different from control siRNA determined by *t*-test.

α-TEA mediated increases in DR5 and CHOP protein levels are JNK dependent and CHOP influences JNK phosphorylation status

Since prior studies showed that JNK activation is a critical component in α-TEA induced apoptosis and other studies have shown that JNK is involved in upregulation of CHOP protein expression [Zou et al., 2008] the possible involvement of JNK in α-TEA mediated increases in CHOP/DR5 protein expression was investigated in MCF-7 cells. siRNA to JNK reduced total JNK and reduced the ability of α-TEA to increase phosphorylated JNK2/1 (pJNK), increase CHOP and DR5 (L/S) protein levels and to induce apoptosis as measured by PARP cleavage (Fig 12A), suggesting that JNK is a critical contributor to increases in CHOP and DR5 (L/S) protein level. Interestingly, siRNA to CHOP reduced α-TEA mediated increases in pJNK2/1 protein levels (Fig 12B), suggesting that α-TEA mediated increases in JNK phosphorylation are dependent on CHOP expression.

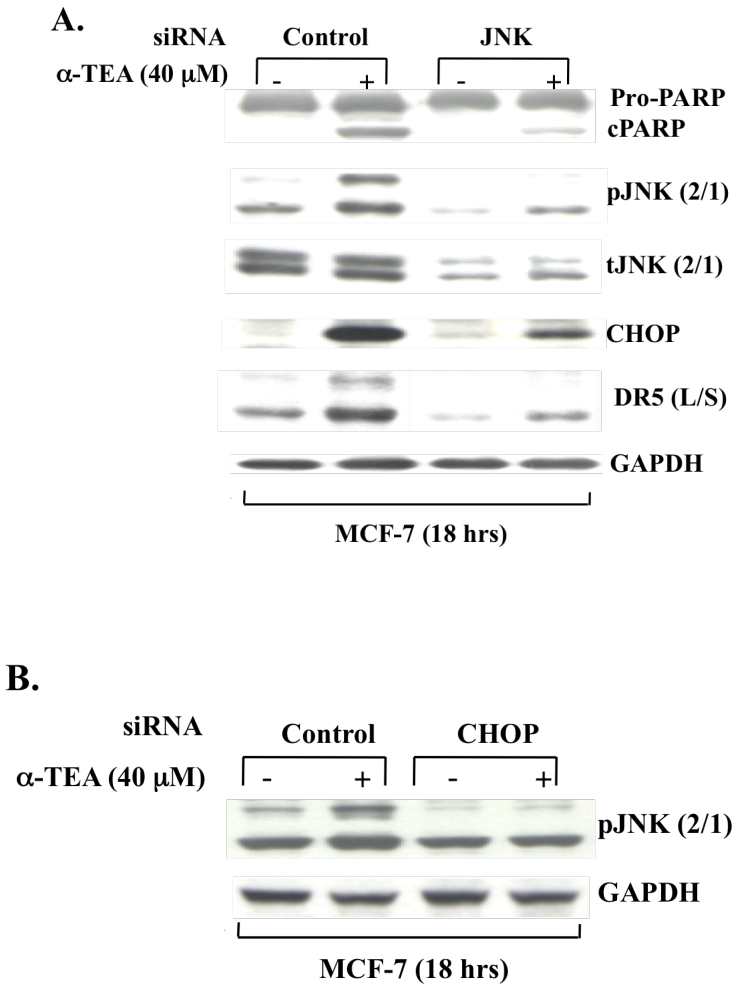
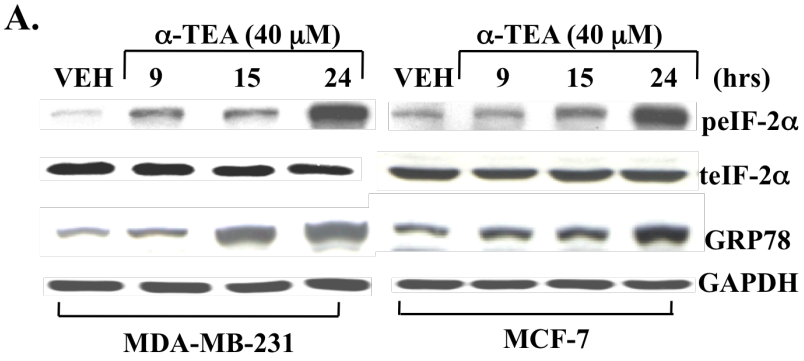
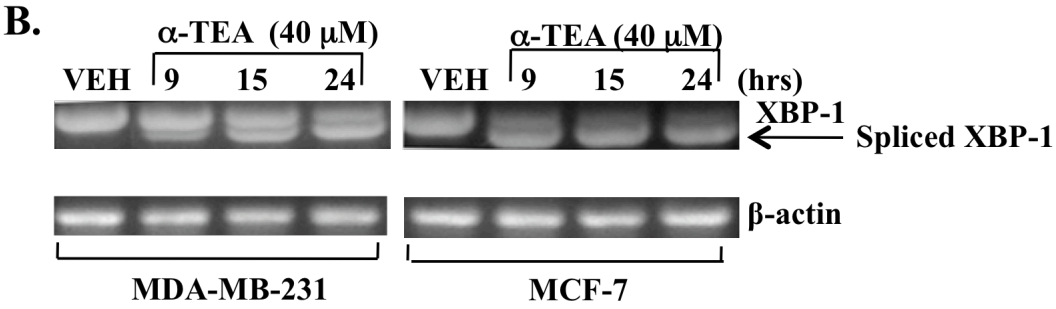


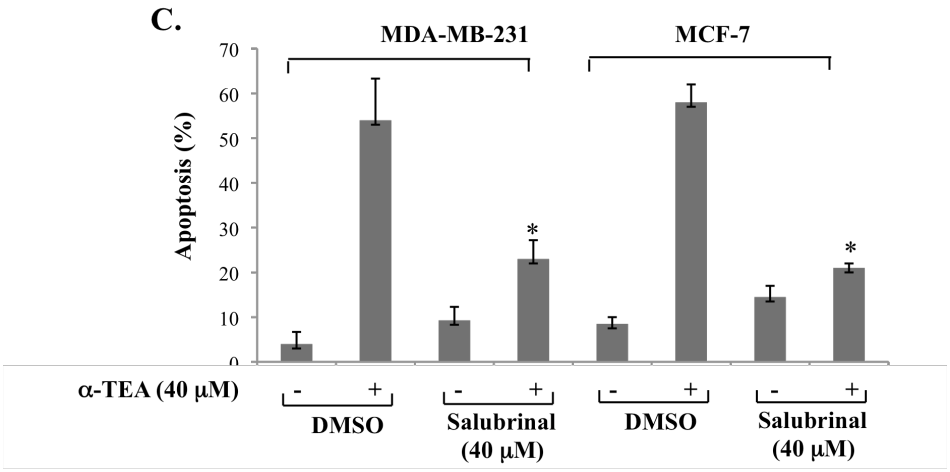
Figure 12. α-TEA induced increased CHOP and DR5 (L/S) protein levels are JNK dependent. In turn, CHOP regulates pJNK2/1 protein levels. A. MCF-7 cells were transiently transfected with siRNA to JNK using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate PARP cleavage, pJNK2/1, total JNK2/1, CHOP, and DR5 (L/S) protein levels using GAPDH as loading control. B. MCF-7 cells were transiently transfected with siRNA to CHOP using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate pJNK2/1 protein levels using GAPDH as loading control. Data from A and B are representative of two or more independent experiments.

Endoplasmic reticulum stress involvement in α-TEA induced apoptosis contributes to JNK/CHOP/DR5 upregulation

α-TEA treatment of MDA-MB-231 and MCF-7 cells increased levels of endoplasmic reticulum stress (ER-stress) indicators: phosphorylated eukaryotic initiation factor 2-alpha (peIF-2α) and glucose-regulated protein of 78kDA (GRP78) also known as BiP (Fig 13A), as well as spliced mRNA forms of X-box binding protein-1 (XBP-1) (Fig 13B) in a time-dependent manner, suggesting that α-TEA treatment induced ER stress in both cell lines. Furthermore, ER-stress inhibitor salubrinal significantly reduced the ability of α-TEA to induce apoptosis detected by Annexin V/FACS (Fig 13C). Since JNK/CHOP/DR5 pathway can be activated in response to ER stress [Zou et al., 2004], studies were conducted to examine if α-TEA mediated JNK/CHOP/DR5 (L/S) signaling events via ER stress. Data show that ER-stress inhibitor salubrinal significantly reduced the ability of α-TEA to upregulate pJNK2/1, CHOP, and DR5 (L/S), as well as to induce PARP cleavage in both cell lines (Fig. 13D). Salubrinal significantly reduced the ability of α-TEA to upregulate ER stress markers GRP78 and peIF2α, confirming the efficiency of ER-stress inhibitor. These data demonstrate that α-TEA induction of JNK/CHOP/DR5 and apoptosis is mediated via ER stress.


A.
α-TEA (40 μM)
α-TEA (40 μM)
VEH
9
15
24
VEH
9
15
24
(hrs)
peIF-2α
teIF-2α
GRP78
GAPDH
MDA-MB-231
MCF-7


B.
α-TEA (40 μM)
α-TEA (40 μM)
VEH
9
15
24
VEH
9
15
24
(hrs)
XBP-1
Spliced XBP-1
β-actin
MDA-MB-231
MCF-7


C.
MDA-MB-231
MCF-7
Apoptosis (%)
70
60
50
40
30
20
10
0
*
*
α-TEA (40 μM)
-
+
-
+
-
+
-
+
DMSO
Salubrinal (40 μM)
DMSO
Salubrinal (40 μM)

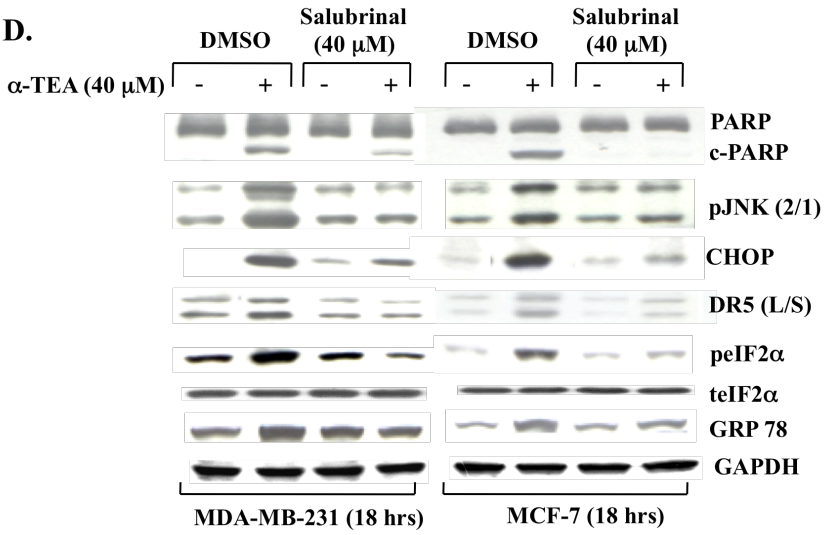
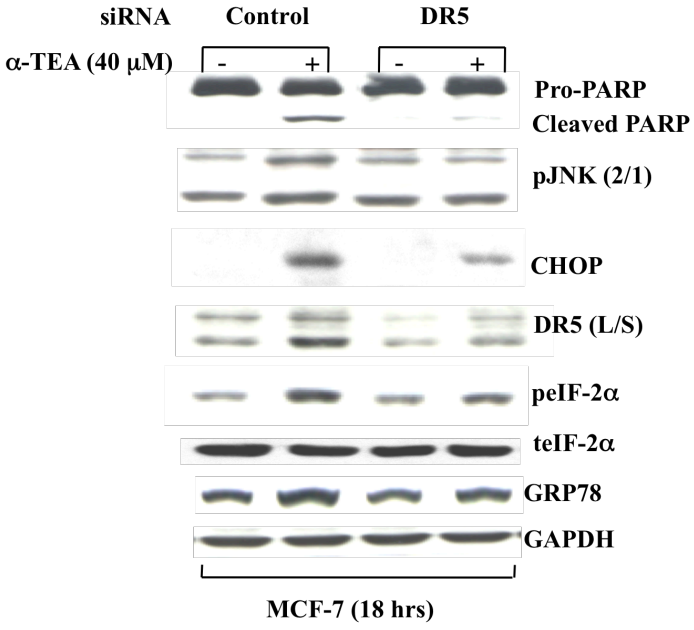


Figure 13. ER stress is involved in α-TEA induced apoptosis and contributes to JNK/CHOP/DR5 upregulation. A. MDA-MB-231 and MCF-7 cells were treated with 40 μM α-TEA for 9, 15, and 24 hrs. Western blot analyses were performed to determine ER stress markers; peIF-2α, total eIF2α and GRP78 protein levels. GAPDH levels were used as lane controls. B. Splicing of XBP-1, a marker for ER stress, was determined by RT-PCR. C. Both cell lines were treated with ER-stress inhibitor salubrinal at 40 μM plus 40 μM α-TEA for 18 hrs. Apoptosis was determined by Annexin V/FACS. D. Western blotting was conducted to evaluate salubrinal effects on α-TEA induced cleavage of PARP, upregulation of pJNK/2/1, CHOP, DR5 (L/S), peIF2α, total eIF2α, and GRP78 proteins. Data from A, B and D are representative of at least 2 individual experiments. Data from C are presented as the mean ± S.D. of three independent experiments. * $P<0.05$= ER-stress inhibitor salubrinal at 40 μM plus 40 μM α-TEA treatment is significantly different from 40 μM α-TEA determined by *t*-test.

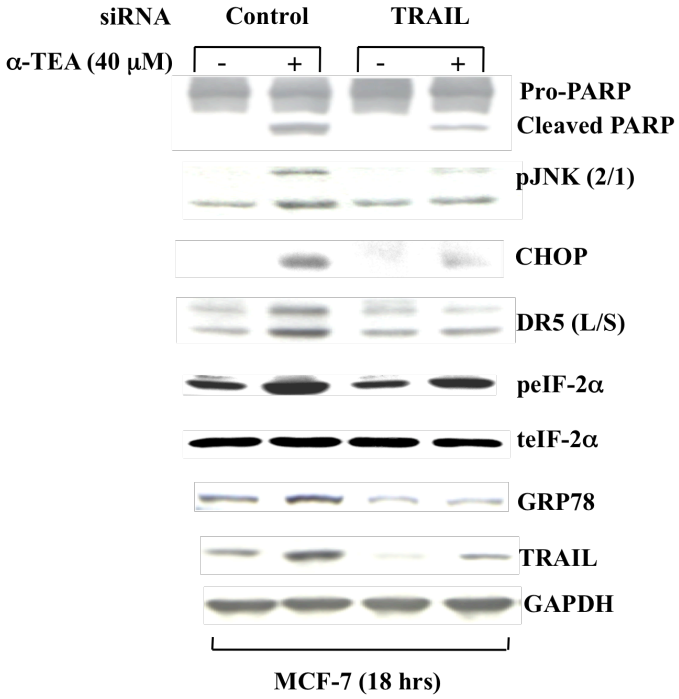
α-TEA induction of ER stress-dependent JNK/CHOP/DR5 signaling is mediated by TRAIL/DR5 pathway

Since both JNK and DR5 can be regulated by TRAIL/DR5 signaling [Shetty et al., 2002], studies were conducted to determine if α-TEA mediated ER stress events are downstream events of TRAIL/DR5 using siRNA knockdown procedures. MCF-7 and MDA-MB-231 cells transiently transfected with siRNAs to DR5 or TRAIL exhibited reduced levels of DR5 and TRAIL, respectively, and showed blockage of α-TEA's ability to produce PARP cleavage and to increase levels of pJNK2/1, CHOP, DR5 (L/S), as well as blocked α-TEA's ability to upregulate ER stress markers GRP78 and peIF-2α (Fig 14 A, B & C), demonstrating that α-TEA induction of ER stress-dependent JNK/CHOP/DR5 signaling is mediated by TRAIL/DR5 pathway.

A.


siRNA
Control
DR5
α-TEA (40 μM)
-
+
-
+
Pro-PARP
Cleaved PARP
pJNK (2/1)
CHOP
DR5 (L/S)
peIF-2α
teIF-2α
GRP78
GAPDH
MCF-7 (18 hrs)

B.


siRNA
Control
TRAIL
α-TEA (40 μM)
-
+
-
+
Pro-PARP
Cleaved PARP
pJNK (2/1)
CHOP
DR5 (L/S)
peIF-2α
teIF-2α
GRP78
TRAIL
GAPDH
MCF-7 (18 hrs)

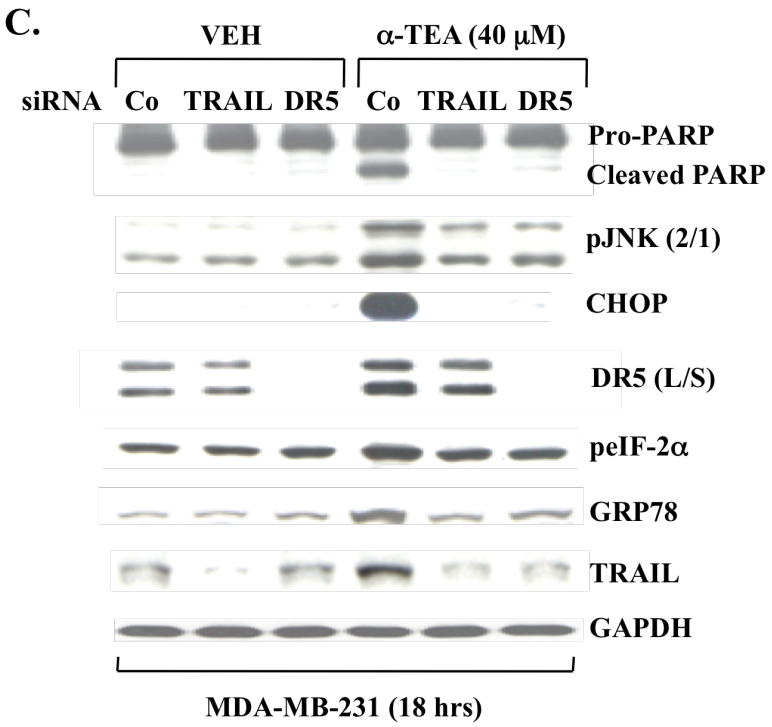


Figure 14. ER stress dependent JNK/CHOP/DR5 upregulation is mediated by TRAIL/DR5 pathway in α-TEA induced apoptosis. A & B. MCF-7 cells were transiently transfected with siRNA to DR5 or TRAIL, using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate PARP cleavage, pJNK2/1, CHOP, DR5 (L/S), peIF-2α total eIF-2α and GRP78 protein levels, using GAPDH as loading control for (A & B) and TRAIL protein levels for siRNA knockdown efficiency (B). C. MDA-MB-231 cells were transiently transfected with siRNA to DR5 or TRAIL, using non-specific siRNA as negative controls followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate PARP cleavage, pJNK (2/1), CHOP, DR5 (L/S), peIF-2α , GRP78, and TRAIL protein levels, using GAPDH as loading control. Data from A, B and C are representative of at least 2 individual experiments.

ER stress dependent JNK/CHOP/DR5 upregulation occurs both upstream and downstream of caspase-8 activation. In an effort to determine if ER stress proceeds or follows α-TEA activation of DR5 signaling, caspase-8 inhibitor (Z-IETD-FMK) was employed. Data show that caspase-8 inhibitor significantly reduced α-TEA abilities to induce apoptosis (Fig 15A), and cleavage of Bid, caspase-9 and PARP, as well as increase protein levels of pJNK2/1, CHOP, DR5, and ER stress markers GRP78 and peIF-2α in both cell types (Fig 15B). These data suggest that α-TEA activation of caspase-8 is upstream of ER-stress mediated JNK/CHOP/DR5 signaling. siRNA knockdown of JNK, CHOP and DR5 in MCF-7 cells reduced the ability of α-TEA to cleave caspases 8 and 9 (Fig 15 C), suggesting that not only DR5 but also JNK and CHOP are involved in activation of caspases-8 and -9. Furthermore, treatment of MDA-MB-231 and MCF-7 cells with ER inhibitor salubrinal blocked α-TEA's ability to induce caspases-8 and -9 cleavage (Fig 15 D), providing additional evidence for caspase 8 acting prior to and after α-TEA induced ER stress.

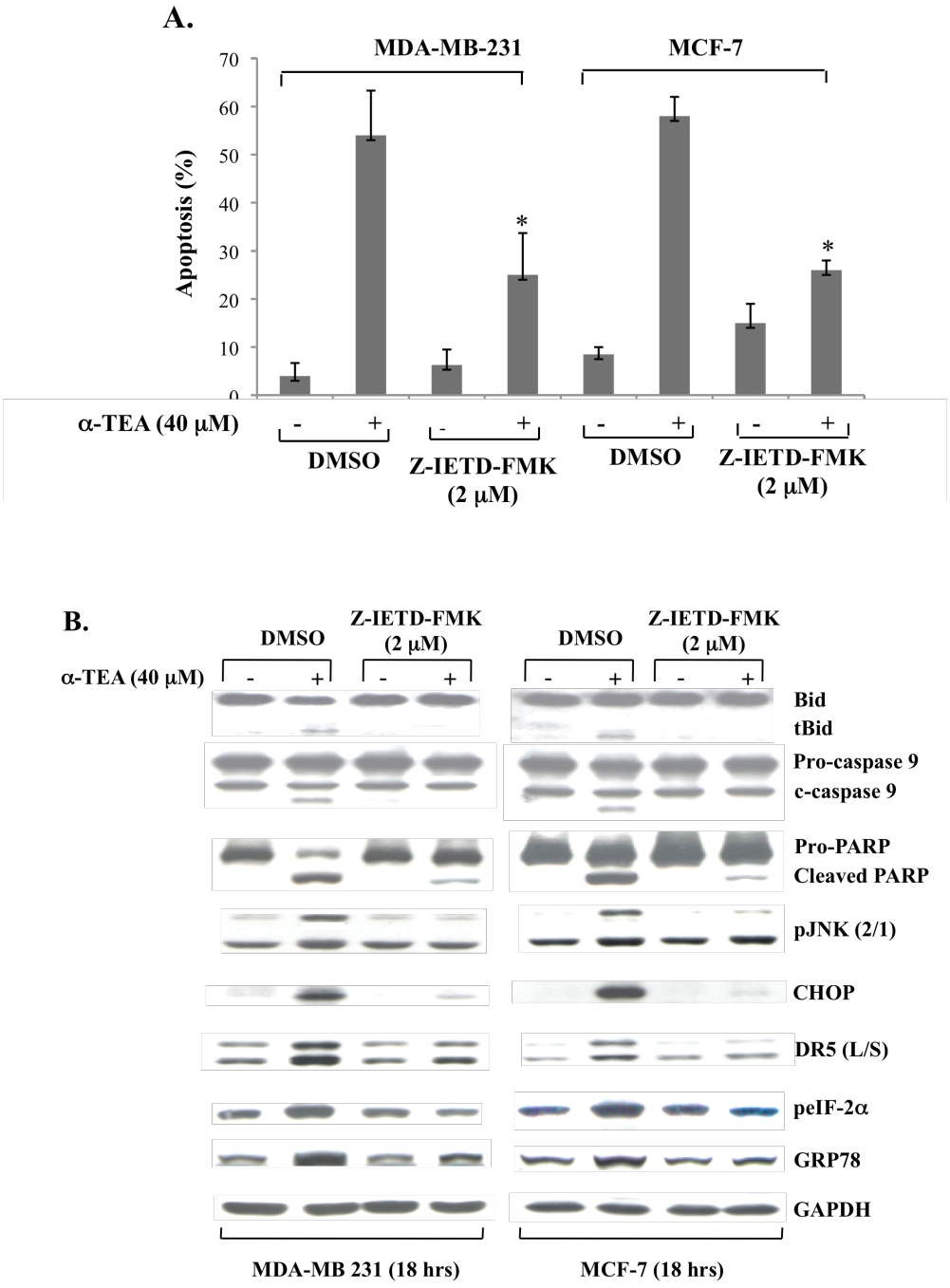


Figure 15. ER stress-dependent JNK/CHOP/DR5 upregulation is both upstream and downstream events of caspase-8.

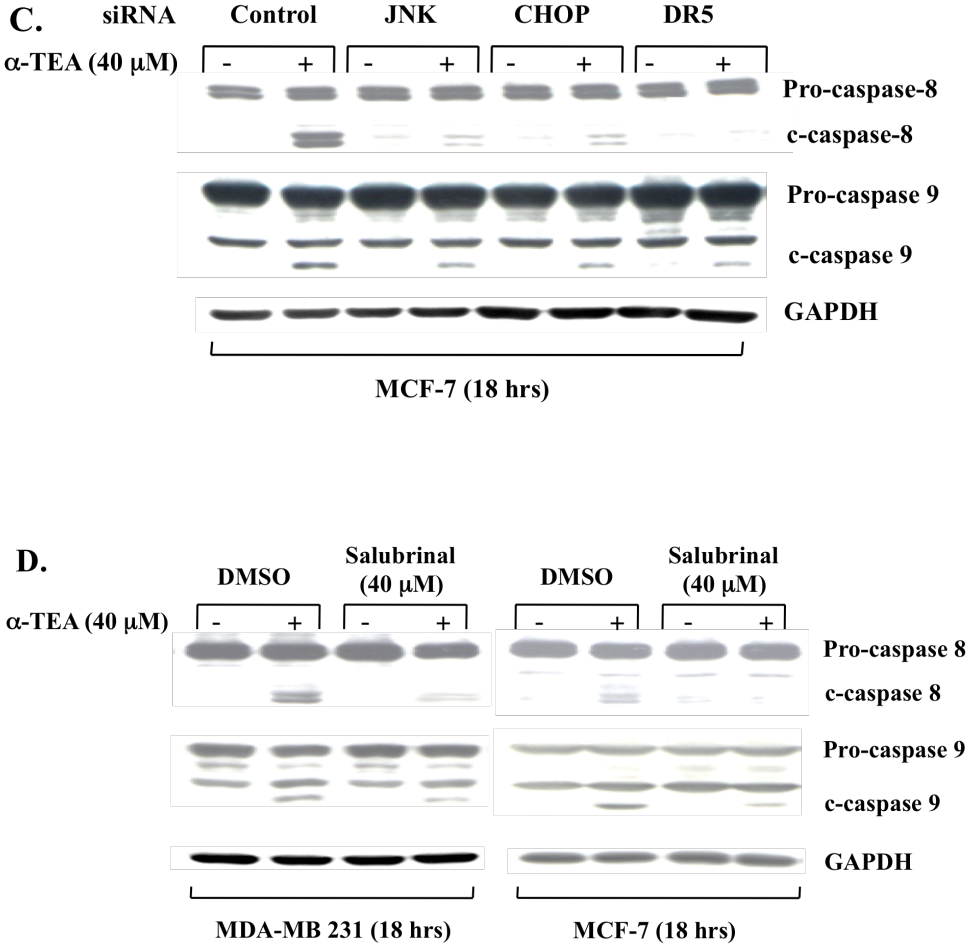


Figure 15. ER stress-dependent JNK/CHOP/DR5 upregulation is both upstream and downstream events of caspase-8. A. MDA-MB-231 and MCF-7 cells were cultured with caspase-8 inhibitor (Z-IETD-FMK) or DMSO vehicle control plus 40 μM α-TEA for 18 hrs. Apoptosis was determined by Annexin V/FACS. B. Samples (from A) were analyzed by western blot to evaluate caspase-8 cleavage of Bid to tBid, cleaved caspase 9 and PARP, pJNK2/1, CHOP, DR5 (L/S), peIF-2α and GRP78 protein levels using GAPDH as loading control. C. MCF-7 cells were transiently transfected with siRNA to JNK, CHOP and DR5, using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate caspase-8 and -9 cleavages, GAPDH served as lane controls. D. MDA-MB-231 and MCF-7 cells were cultured with ER-stress inhibitor salubrinal at 40 μM plus 40 μM α-TEA for 18 hrs. Western blot was conducted to evaluate caspase-8 and -9 cleavages, GAPDH served as lane controls. Data from B, C and D are representative of two or more individual experiments. Data from A are presented as the mean ± S.D. of three independent experiments. * $P<0.05$= caspase-8 inhibitor (Z-IETD-FMK) at 2 μM plus 40 μM α-TEA treatment is significantly different from 40 μM α-TEA determined by *t*-test.

α-TEA down regulation of c-FLIP (L) protein levels is in part, mediated by caspase-8/ER stress/JNK/CHOP/DR5 signaling via Itch E3 ligase ubiquitination. α-TEA downregulates c-FLIP (L) protein levels in both MDA-MB-231 and MCF-7 breast cancer cells in a time dependent manner (Fig 16A). MCF-7 cells transiently transfected with siRNA to CHOP, JNK or DR5, and both MDA-MB-231 and MCF-7 cells cultured with ER-stress inhibitor salubrinal or caspase 8 inhibitor Z-IETD-FMK prevented the ability of α-TEA to decrease c-FLIP (L) protein levels (Fig 16B and C), providing evidence that α-TEA downregulation of c-FLIP is mediated by caspase-8/ER stress/JNK/CHOP/DR5 signaling**.** Since c-FLIP has been reported to be degraded via JNK-dependent E3 ubiquitin ligase Itch [Chang et al., 2006], studies were conducted to determine if Itch E3 ligase mediated ubiquitination was involved in α-TEA down-regulation of c-FLIP. Knockdown of Itch E3 ligase with siRNA to Itch reduced α-TEA's ability to downregulate c-FLIP (L) protein levels in MCF-7 cells (Fig. 16D). siRNA to Itch significantly reduced the ability of α-TEA to induce apoptosis in MDA-MB-231 and MCF-7 cells (Fig 16E). These data suggest that α-TEA induced downregulation of c-FLIP (L) is mediated by a caspase-8/ER stress/JNK/CHOP/DR5 pathway via Itch ligase ubiquitination.

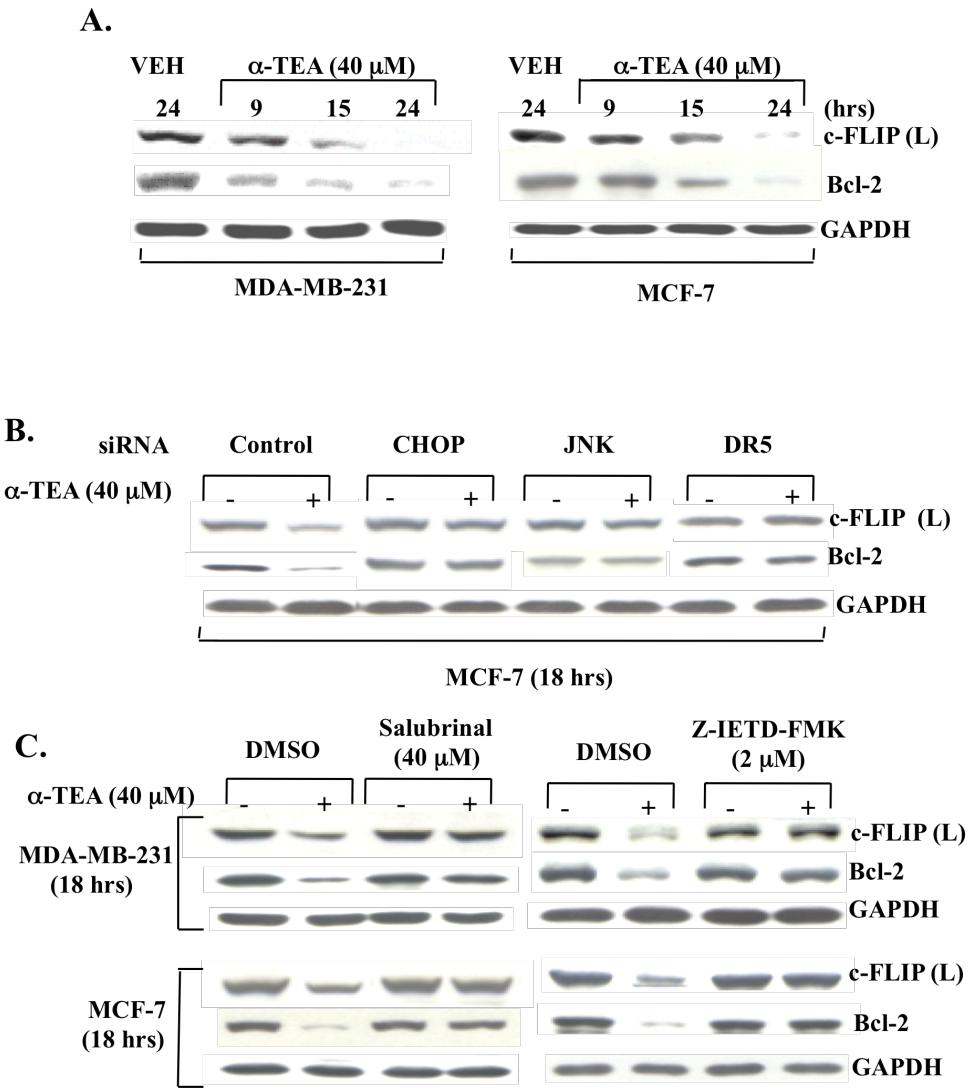
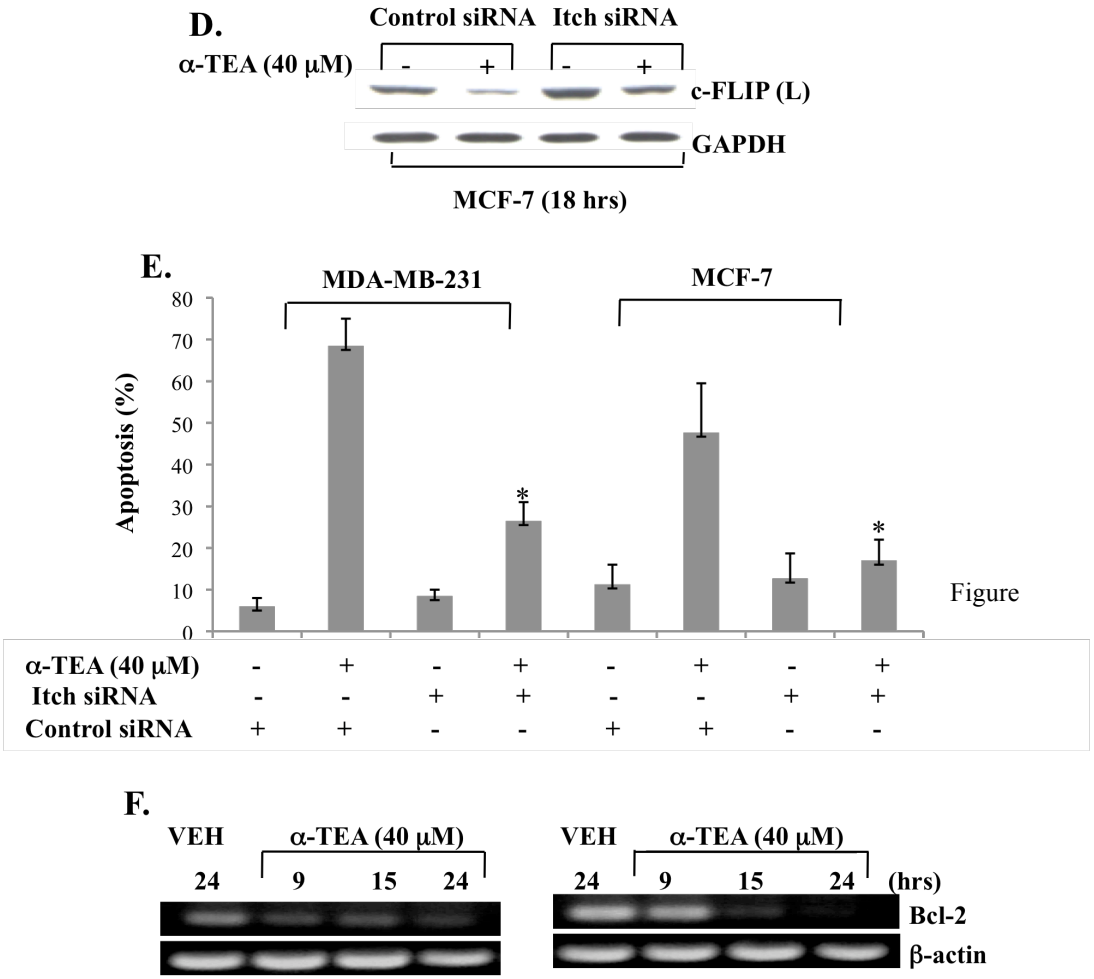


Figure 16. α-TEA decreased Bcl-2 and c-FLIP (L) protein levels via caspase-8 dependent ER stress-mediated JNK/CHOP/DR5 pathway. A. MDA-MB-231 and MCF-7 cells were treated with 40 μM α-TEA for 9, 15, and 24 hrs. Western blot analyses were performed to evaluate Bcl-2 and c-FLIP (L) protein levels with GADPH as loading control. B. MCF-7 cells were transiently transfected with JNK, CHOP, and DR5 siRNA or control siRNA followed by treatment with 40 μM α-TEA or vehicle for 18 hrs. Bcl-2 and c-FLIP (L) protein levels were determined by western blot analyses, using GAPDH as loading control. C. MDA-MB-231 and MCF-7 cells were cultured with ER-stress inhibitor salubrinal at 40 μM or caspase-8 inhibitor (Z-IETD-FMK) at 2 μM plus 40 μM α-TEA for 18 hrs. Bcl-2 and c-FLIP (L) protein levels were determined by western blot analyses, using GAPDH as loading control.



Figure

16. α-TEA decreased Bcl-2 and c-FLIP (L) protein levels via caspase-8 dependent ER stress-mediated JNK/CHOP/DR5 pathway. D. MCF-7 cells were transiently transfected with Itch siRNA or control siRNA followed by treatment with 40 μM α-TEA or vehicle for 18 hrs. c-FLIP (L) protein levels were determined by western blot analyses. E. MDA-MB-231 and MCF-7 cells were transiently transfected with siRNA to itch using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Apoptosis was determined by Annexin V/FACS. F. MDA-MB-231 and MCF-7 cells were treated with 40 μM α-TEA for 9, 15, and 24 hrs. mRNA levels of Bcl-2 were determined by RT-PCR. Data from E are the mean ± S.D. of three independent experiments. * $p<0.05$=significantly different from control siRNA determined by *t*-test.

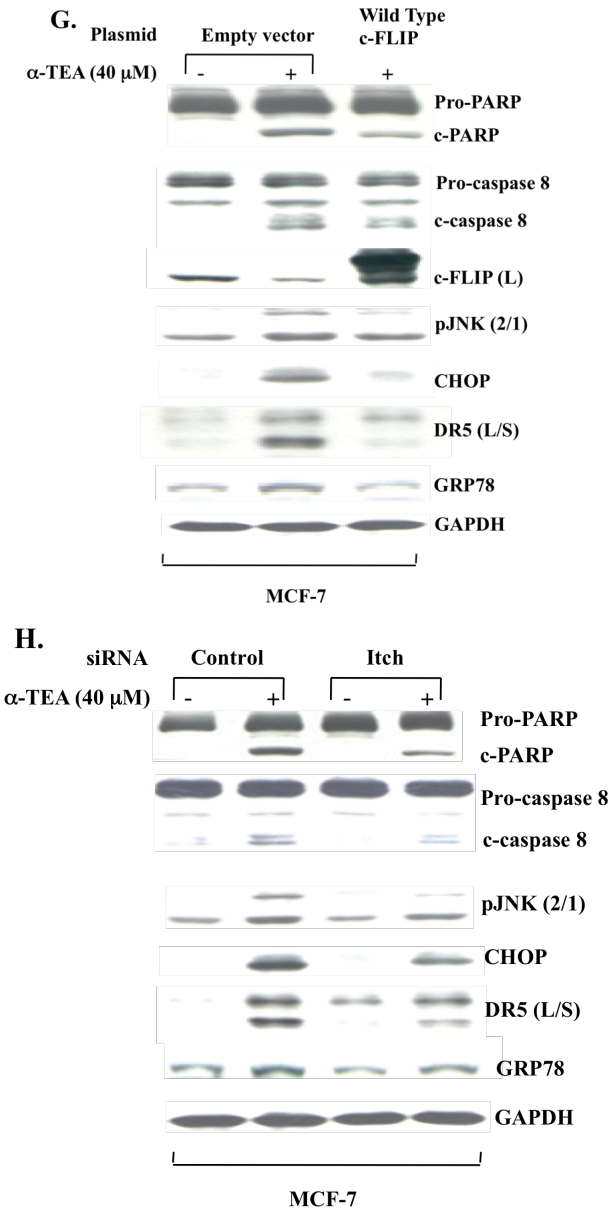


Figure 16. α-TEA decreased Bcl-2 and c-FLIP (L) protein levels via caspase-8 dependent ER stress-mediated JNK/CHOP/DR5 pathway. G. MCF-7 cells were transiently transfected with wild-type c-FLIP plasmid or vector control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate over expression of c-FLIP on ability of α-TEA to cleave PARP and caspase-8, and down-regulate c-FLIP (L), pJNK2/1, CHOP, DR5 (L/S), peIF-2α and GRP78 protein expression using GAPDH as loading control. H. MCF-7 cells were transiently transfected with siRNA to Itch, using non-specific siRNA as negative control followed by treatment with 40 μM α-TEA for 18 hrs. Western blot analyses were performed to evaluate elevated levels of c-FLIP (L) on ability of α-TEA to cleave PARP and caspase-8 and to down-regulate pJNK2/1, CHOP, DR5 (L/S) and GRP78 protein levels using GAPDH as loading control. Data for A, B, C, D, F, G and H are representative of two or more individual experiments.

α-TEA downregulation of c-FLIP mediates ER stress-dependent JNK/CHOP/DR5 signaling via activation of caspase-8

Since c-FLIP is an inhibitor of caspase-8 activation, we hypothesized that α-TEA downregulation of c-FLIP may contribute to activation of caspase-8 and subsequent ER stress-dependent JNK/CHOP/DR5 signaling. To test this hypothesis, MCF-7 cells were transiently transfected with wildtype c-FLIP (L) plasmid or Itch siRNA followed by treatment with α-TEA (40 μM) for 18 hrs. Over-expression of c-FLIP enhanced c-FLIP (L) levels (Fig 16G) and siRNA to itch blocked α-TEA's ability to reduce c-FLIP protein expression (Fig 16D). Both overexpression of c-FLIP (L) protein and restoration of c-FLIP protein expression by silencing itch in α-TEA treatment reduced the ability of α-TEA to cleave PARP and caspase-8, and to increase protein levels of pJNK2/1, CHOP, DR5, and GRP 78 (Fig 16G and H). These data suggest that α-TEA's ability to downregulate c-FLIP (L) is important for caspase-8 activation and subsequent activation of the ER stress/JNK/CHOP/DR5 signaling.

α-TEA downregulation of Bcl-2 protein and mRNA expression is mediated by ER stress

α-TEA treatment of MDA-MB-231 and MCF-7 cells reduced both Bcl-2 protein and mRNA levels in a time dependent manner (Fig 16A and F). siRNA to JNK, CHOP, and DR5 (Fig 16B) as well as caspase-8 and ER stress inhibitors (Fig 16C) blocked the ability of α-TEA to decrease Bcl-2 protein expression. These data suggest that α-TEA induced down-regulation of Bcl-2 is mediated by caspase-8, ER stress, JNK, CHOP and DR5.

2.4. Discussion

The underlying mechanisms of how α-TEA, a potential chemotherapeutic drug, enhances DR5 death signaling pathways were investigated. For the first time, studies demonstrate that: (i) α-TEA induces ER stress dependent apoptosis, (ii) α-TEA induced ER stress regulates JNK/CHOP/DR5 signaling and is mediated by TRAIL/DR5/caspase-8, forming a positive loop, (iii) α-TEA downregulation of c-FLIP is mediated by ER stress-dependent JNK/CHOP/DR5 signaling via JNK activation of Itch E3 ligase ubiquitination and involved in activation of the ER-stress-dependent events via reducing the inhibitory effect of c-FLIP on caspase-8, and (iv) α-TEA downregulation of Bcl-2 protein levels is mediated by the ER stress-dependent events. Taken together, these data show that α-TEA induces TRAIL/DR5/caspase-8 dependent ER stress that triggers JNK/CHOP/DR5 signaling enhancing DR5/caspase-8 mediated mitochondria-dependent apoptotic signaling and produces a downregulation of two key anti-apoptotic factors; c-FLIP and Bcl-2. Based on these data, we provide a schematic diagram depicting a series of events including a positive-acting feedback loop, which is operative in α-TEA induced DR5 dependent apoptosis of human breast cancer cells in culture (Fig 17). DR5 signaling triggers caspase-8 activation, leading to two α-TEA initiated pathways: (i) a classic mitochondria-dependent apoptotic cascade initiated by cleavage of Bid and (ii) an ER-stress dependent activation of JNK/CHOP/DR5 sequence leading to further activation of caspase-8

and downregulation of anti-apoptotic factors; c-FLIP and Bcl-2. Importantly, down-regulation of c-FLIP serves to further enhance caspase-8 activation, which sustains the ER-stress/JNK/CHOP/DR5 amplifying loop.

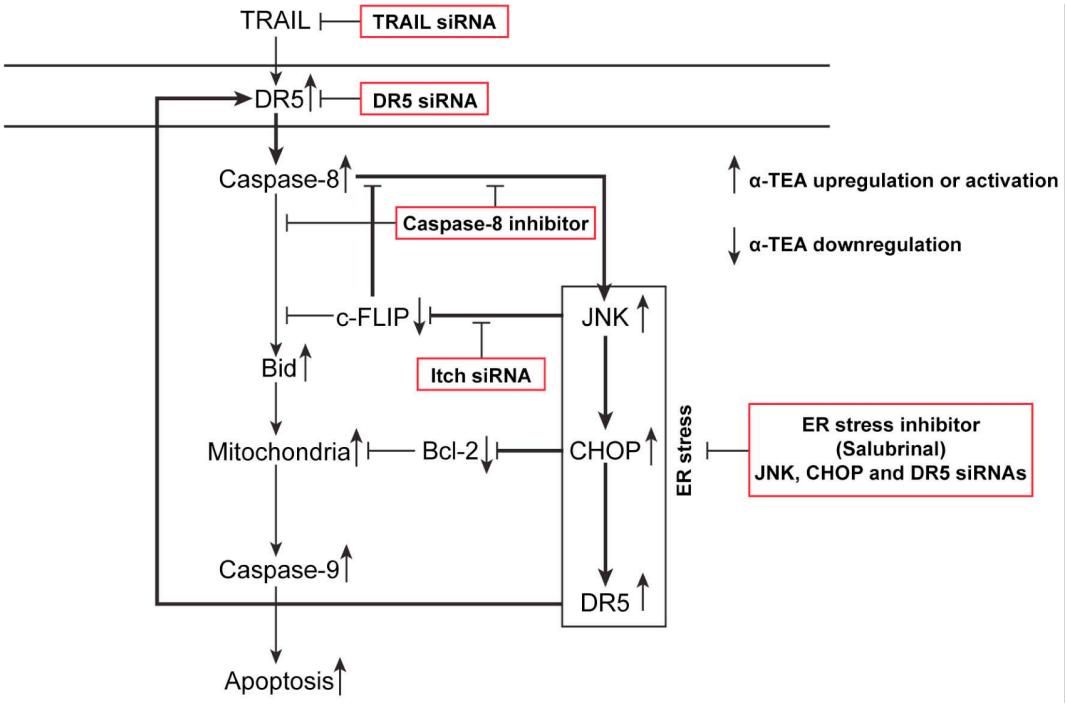


Figure 17. Schematic diagram showing α-TEA to induce breast cancer cells to undergo apoptosis via caspase-8 mediated ER stress JNK/CHOP/DR5 amplification loop and by down-regulation of anti-apoptotic mediators c-FLIP (L) and Bcl-2.

TRAIL has emerged as a potent anticancer agent based on its ability to induce apoptosis in tumor cells but not in most normal cells [Ashkenazi, 2008; Kruyt, 2008]. Thus, targeting TRAIL/DR4/DR5 signaling pathways holds promise for inducing pro-apoptotic signaling in many cancer types while sparing normal cells and tissues [Elrod et al., 2008]. Although considerable effort has been made in

investigating the biological ramifications of TRAIL death receptor signaling, a complete understanding of the multiple pathways and events involved remain unclear. Typically, signaling pathways that initiate apoptosis have been broadly classified as (i) extrinsic death receptor initiated pathways or (ii) intrinsic pathways initiated by mitochondrial events [Elmore, 2007]. These two pathways are not mutually exclusive, and JNK and caspase-8 have been observed to play central roles in both [Dhanasekaran et al., 2008]. Our findings that TRAIL/DR5/casapse-8 triggers an ER stress dependent JNK/CHOP/DR5 amplifying loop and a JNK/c-FLIP/caspase-8 amplifying loop in α-TEA induced apoptosis provides new insights into TRAIL/DR5-mediated apoptotic signaling.

DR5 upregulation has been reported to be mediated by NF-kB in TRAIL-induced apoptosis in MCF-7 and MDA-MB-231 cells [Shetty et al., 2002]. However, inhibition of NF-kB using IkB phosphorylation inhibitor BAY-11-7085 [Hernández-Gutierrez et al., 2006] enhances α-TEA induced upregulation of DR5 and apoptosis in MCF-7 cells (data not shown), suggesting that NF-kB plays an anti-apoptotic role in α-TEA-induced apoptosis. Data reported here showing that α-TEA up-regulates DR5 via CHOP binding to the DR5 promoter is supported by published reports showing that CHOP can directly regulate DR5 expression through a CHOP binding site in the 5'-flanking region of the DR5 promoter [Yoshida et al., 2005].

JNK is a stress responsive kinase that has been reported to induce CHOP expression via an AP-1 binding site in the CHOP promoter [Guyton et al., 1996]. JNK has been shown to be activated and is required for α-TEA-induced apoptosis of human breast, ovarian and prostate cancer cells [Jia et al., 2008; Wang et al., 2008]. Data presented here show that siRNA to JNK blocks α-TEA induced CHOP as well as DR5, indicating that CHOP/DR5 upregulation is mediated via JNK in α-TEA induced apoptosis. These data are in agreement with other studies showing that CHOP is an ER stress-regulated protein [Oyadomari et al., 2004], JNK activation can contribute to CHOP expression during ER stress [Li et al., 2004], and coupling of JNK/CHOP/DR5 are involved in ER stress-mediated apoptosis.

Present study show that α-TEA induces increased levels of peIF-2α and GRP78 proteins and spliced XBP-1, all of which are recognized as ER stress markers [Szegezdi et al., 2006] and that salubrinal, a selective inhibitor of eIF-2α dephosphorylation (protects cells from ER stress) blocked α-TEA induced apoptosis and the ability of α-TEA to activate JNK/CHOP/DR5 and upregulate ER stress markers, confirming that ER stress is induced by α-TEA and is involved in both apoptosis and activation of JNK/CHOP/DR5.

Present data show that α-TEA induction of the ER stress dependent JNK/CHOP/DR5 pathway is a downstream consequence of TRAIL/DR5 signaling. Furthermore, a caspase-8 inhibitor not only blocks α-TEA induced

mitochondria-dependent apoptotic cascade as indicated by blockage of Bid and pro-caspase-9 cleavage, but also blocks ER stress signaling events, indicating that TRAIL/DR5 downstream effector caspase-8 contributes to α-TEA induced ER stress-dependent events. To our knowledge, this is the first report that ER stress can be triggered by a TRAIL/DR5/caspase-8 pathway and that ER stress induces a JNK/CHOP/DR5 amplifying loop that can further enhance TRAIL/DR5-mediated apoptosis. The mechanism(s) whereby activated caspase-8 induces ER stress were not investigated in this study. However, studies using hepatoblastoma HepG2 cells suggest that activated caspase-8 may promote and amplify the ER stress response by cleaving BAP31, an integral membrane protein of the ER, in glycochenodeoxycholic acid-induced apoptosis [Lizaka et al., 2007].

As a caspase-8 inhibitor, c-FLIP plays an important role in death receptor dependent apoptosis. Published data showed that down-regulation of c-FLIP is involved in α-TEA induced apoptosis in human ovarian and prostate cancer cells. However, how α-TEA regulates c-FLIP is not known. Data reported here show that down-regulation of c-FLIP is mediated by the caspase-8 dependent ER stress JNK/CHOP/DR5 loop via JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch. JNK-mediated protein degradation of c-FLIP has been reported for TNF-induced cell death [Chang et al., 2006]. However, it is the first report that JNK-mediated c-FLIP degradation is ER stress dependent.

Bcl-2 is a well established anti-apoptotic factor, which inhibits mitochondrial dependent apoptosis. It has been reported that ER stress induces downregulation of Bcl-2 via CHOP [Habener et al., 1996]. Although CHOP is a transcription factor, a CHOP responsive site in the Bcl-2 promoter has not been reported; suggesting that if CHOP mediates transcriptional regulation of Bcl-2 expression it must form a complex with other proteins [49]. Here, for the first time, we demonstrate that α-TEA downregulates Bcl-2 at both protein and mRNA levels, which is regulated by ER-stress mediated JNK/CHOP/DR5 signaling.

In conclusion, data show that α-TEA induces a TRAIL/DR5/caspase-8 dependent ER stress-mediated JNK/CHOP/DR5 loop, which amplifies caspase-8-mediated mitochondrial-dependent apoptosis via downregulation of c-FLIP and Bcl-2. These data are significant in that they provide a better understanding of the pro-apoptotic actions of the anti-cancer agent α-TEA, and provide new insights into DR5-mediated apoptotic signaling in human breast cancer cells.

Chapter 3. Vitamin E analog α-TEA cooperates with MEK or mTOR inhibitors to induce apoptosis via targeting IRS-1/PI3K pathways

3.1. Introduction

The PI3K, AKT, ERK, and mTOR pro-survival mediators are important therapeutic targets since they are constitutively activated in many cancers and contribute to cancer progression by promoting cellular proliferation and inhibiting cell death signaling pathways (Falasca, 2010). PI3K is activated at the cell membrane by tyrosine kinase growth factor receptors such as members of the epidermal growth factor receptor (EGFR) family and by the insulin-like growth factor-1 receptor (IGFR), as well as its down-stream signaling substrate IRS-1 (Schlessinger, 2000). PI3K promotes cancer cell survival by activation of downstream mediators AKT and Ras, the latter leading to ERK activation (McCubrey et al, 2007). AKT exerts its survival role via a diverse array of substrates, which control key cellular processes, including apoptosis, cell cycle progression, transcription, and translation (Chang et al, 2003). A major downstream substrate of AKT is the serine/threonine kinase mTOR. AKT can directly phosphorylate mTOR at ser-2448 and activate it, as well as cause indirect activation of mTOR by phosphorylating and inactivating tuberous sclerosis

complex 2 (TSC2), also called tuberin). The Raptor-mTOR-complex signals to its downstream effectors S6 kinase/ribosomal protein S6 (p70S6K) and the eIF4E binding protein (p4E-BP1**)** to control transcription and translation, which selectively regulates multiple proteins that control cell cycle and apoptosis (Gibbons et al, 2009). Additionally, AKT can directly regulate apoptosis by phosphorylating and inactivating pro-apoptotic proteins such as Bad and caspase-9 (Datta et al, 1997; Cardone et al, 1998; Mabuchi et al, 2002). ERK exerts its anti-apoptotic effects by phosphorylating and inactivating Bad (Mabuchi et al, 2002). As with most intracellular signaling cascades, cross-talk and negative and positive feedback loops complicate final signaling outcomes.

For example, the mTOR substrate p70S6K can ultimately diminish pro-survival signaling via PI3K/AKT by catalyzing an inhibitory phosphorylation site on insulin receptor substrate-1, an upstream mediator of PI3K (Wan et al, 2007). Likewise, ERK can diminish pro-survival signaling by PI3K/AKT via p70S6K (Jiang et al, 2009). Therefore, although ERK and mTOR showed potential as anticancer targets, inhibitors of ERK or mTOR alone are limited in clinical application due to the mitigation of these negative feedback loops essential for controlling AKT activity (Sun et al, 2005). Thus, this more in depth understanding of signaling pathways suggests that ERK or mTOR inhibitors need to be combined with agents that can circumvent the loss of negative feedback controls on AKT and/or effectively block AKT activity.

α-TEA is a promising agent for cancer prevention/therapy based on its antitumor actions reported in several *in vitro* and *in vivo* studies of a variety of cancers, including human estrogen-responsive and non-responsive breast cancers (Lawson et al, 2004; Anderson et al, 2002; Hahn et al, 2006; Jia et al, 2008; Yu et al, 2006; Hahn et al, 2009; Shun et al, 2004; Shun et al, 2010; Jia et al, 2008; Wang et al, 2008). These previous studies showed that α-TEA induces apoptosis in human breast cancer cells via activation of pro-apoptotic extrinsic death receptor Fas (CD95/Apo-1) and death receptor 5 (DR5) as well as activation of a JNK/p73/Noxa pathway, leading to activation of caspase-8 and mitochondrial-dependent apoptosis (Shun et al, 2004; Wang et al, 2008; unpublished data). In this study, we demonstrate that α-TEA induces apoptosis not only by activation of pro-apoptotic pathways but also by targeting IRS-1/PI3K growth/survival pathways to suppress multiple pro-survival factors via JNK. Lessons learned from this study are that α-TEA in combination with ERK or mTOR inhibitors is an effective anticancer strategy and lays the groundwork for a mechanism-based combinational therapeutic strategy for employing ERK and mTOR inhibitors more effectively in the clinic.

3.2. Materials and Methods

Chemicals

α-TEA (F.W.= 488.8) was prepared in house as described previously (Lawson et al, 2004). MEK inhibitor (U01260) and PI3K inhibitor (wortmannin) were purchased from Cell Signaling Technology (Beverly, MA). mTOR inhibitor (rapamycin) and JNK inhibitor (SP600125) were purchased from Calbiochem (La Jolla, CA).

Cell Culture

HCC-1954 estrogen-receptor negative human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 estrogen-responsive human breast cancer cells were originally provided by Dr. Suzanne Fuqua (Baylor College of Medicine, Houston, TX). MCF-7 cells were cultured as previously described (Wang et al, 2008). HCC-1954 cells were cultured in RPMI media with 10% fetal bovine serum (FBS). For experiments, FBS was reduced to 2% and cells were allowed to attach overnight before treatments. α-TEA (40 mM) dissolved in ethanol served as stock solution. Equivalent final concentrations of ethanol (0.05-0.1%) for concentration of α-TEA used served as vehicle controls (VEH).

Western Blot Analyses

Whole cell protein lysates and western blot analyses were conducted as described previously (Jia et al, 2008). Proteins at 20-50 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), phospho-JNK (pJNK thr-183/tyr-185), phospho-ERK (pERK thr-202/tyr-204), total ERK and phospho-caspase-9 (pcaspase-9 ser-196) (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-AKT (pAKT ser-473), pAKT (ser-308), total AKT, phospho-glycogen synthase kinase-3 α/β (pGSK-3 α/β ser-21/9), total GSK-3 α/β, phospho-Bad (pBad ser-136), phospho-Bad (pBad ser-112), total Bad, total casapse-9, phospho-p90 ribosomal s6 kinase (pp90RSK ser-380), total p90RSK, phospho-mTOR (pmTOR ser-2448), total mTOR, phospho-p70S6 kinase (pp70S6K thr-389), total p70S6K, phospho-4E-BP1 (p4E-BP1 thr-37/46), total 4E-BP1, phospho-IRS (pIRS ser-307), total IRS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Beverly, MA).

Small interfering RNA (siRNA) transfection

A scrambled RNA duplex that does not target any known genes was used as a nonspecific negative control for RNAi (referred to as control siRNA) (Ambion,

Austin, TX). Transfection of siRNAs to AKT1, AKT2, JNK1/2 or control (Ambion, Austin, TX) was performed in 100 mm^2 cell culture dishes at a density of 2 x 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 2 x 10^6 cells/dish and incubated for 1 day followed by treatments.

Quantification of apoptosis

Apoptosis was quantified by Annexin V-Fluorescein isothiocyanate (FITC)/ propidium iodide (PI) assay following the manufacturer's instructions. The Annexin V-FITC/PI assay measures amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and amount of 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 (i.e. positive for annexin-V fluorescence) were considered to be apoptotic.

Statistical Analysis

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey test for comparison of more than two treatments or a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $P < 0.05$.

3.3 Results

Fig 1.

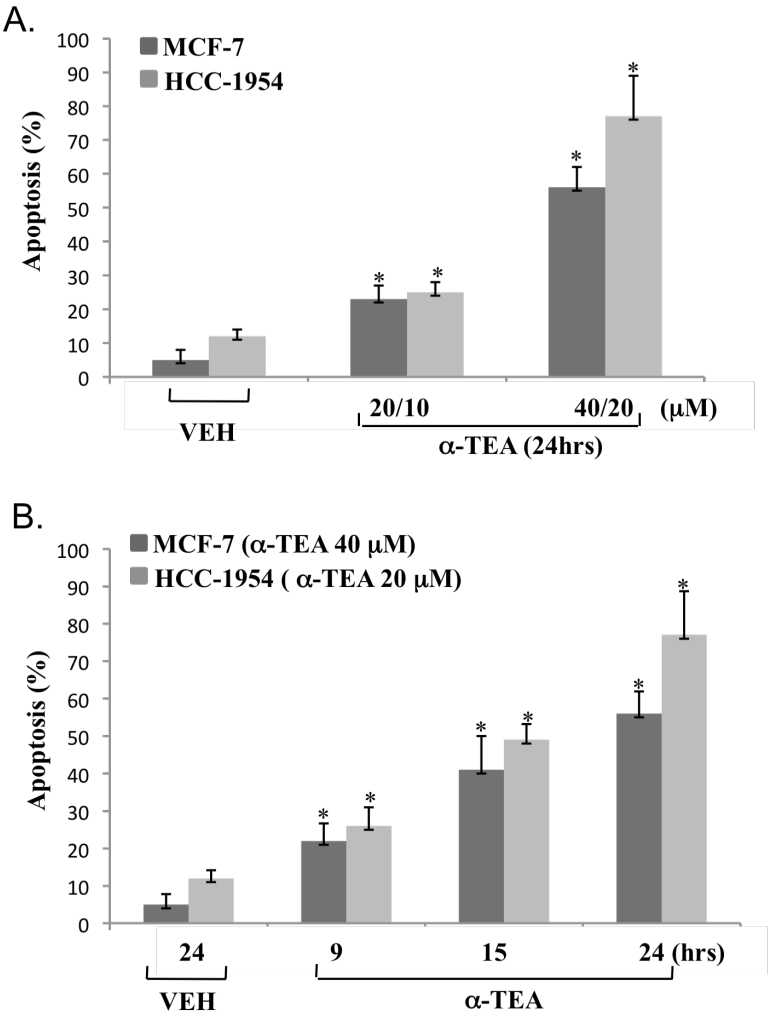
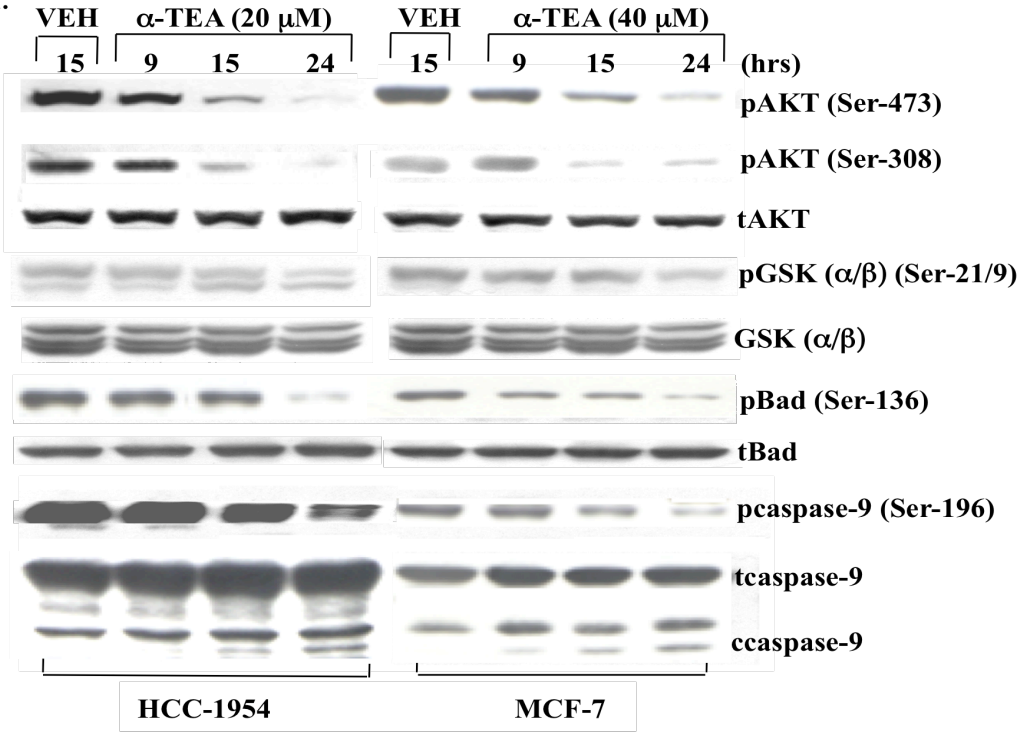


Figure 18. α-TEA induces apoptosis in human breast cancer cells in a dose- and time- dependent manner. (A) MCF-7 and HCC-1954 cells were treated with different concentrations of α-TEA for 24 hrs and the pro-apoptotic property of α-TEA was evaluated by FACS/Annexin V assay. (B) MCF-7 and HCC-1954 cells were treated with 40 and 20 μM α-TEA, respectively, for different times, and the pro-apoptotic property of α-TEA was evaluated by FACS/Annexin V assay. Data are the mean ± S.D. of different independent experiments. *Significantly different from VEH (p<0.05).

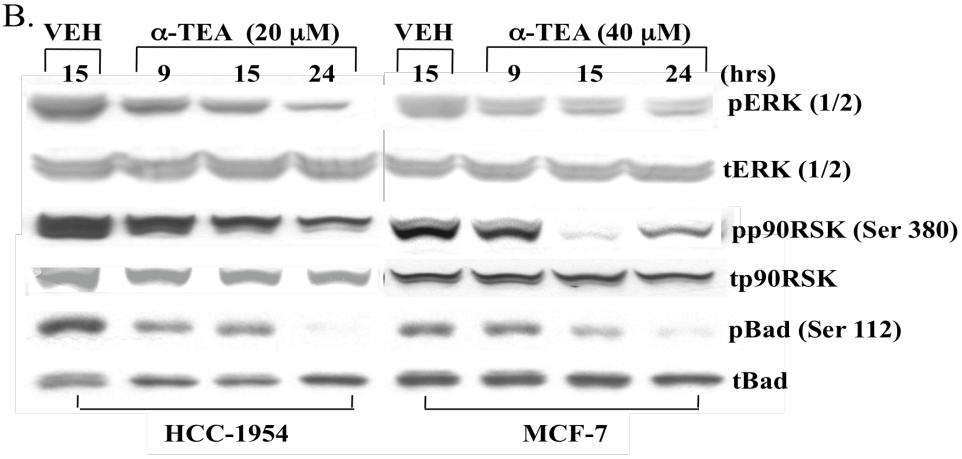
α-TEA induces apoptosis in HCC-1954 and MCF-7 human breast cancer cells

MCF-7 and HCC-1954 human breast cancer cells were treated with α-TEA at different doses (Fig. 18 A) or for different periods of time (Fig. 18 B). Apoptosis was quantified by FACS analyses of cells labeled with FITC-Annexin V. Treatment of MCF-7 cells with α-TEA at 20 or 40 μM and HCC-1954 cells at 10 or 20 μM for 24 hrs induced both cell types to undergo apoptosis in a dose-dependent manner with HCC-1954 cells exhibiting greater sensitivity than MCF-7 cells (Fig. 18 A). Treatment of the MCF-7 and HCC-1954 cells with α-TEA at 40 and 20 μM, respectively, for 9, 15 and 24 hrs induced apoptosis in a time-dependent manner (Fig. 18 B). These data show that α-TEA is a potent apoptotic inducer of both ER-responsive and non-responsive human breast cancer cells.

A.


VEH
α-TEA (20 μM)
VEH
α-TEA (40 μM)
15
9
15
24
15
9
15
24
(hrs)
pAKT (Ser-473)
pAKT (Ser-308)
tAKT
pGSK (α/β) (Ser-21/9)
GSK (α/β)
pBad (Ser-136)
tBad
pcaspase-9 (Ser-196)
tcaspase-9
ccaspase-9
HCC-1954
MCF-7

B.


VEH
α-TEA (20 μM)
VEH
α-TEA (40 μM)
15
9
15
24
15
9
15
24
(hrs)
pERK (1/2)
tERK (1/2)
pp90RSK (Ser 380)
tp90RSK
pBad (Ser 112)
tBad
HCC-1954
MCF-7

C.

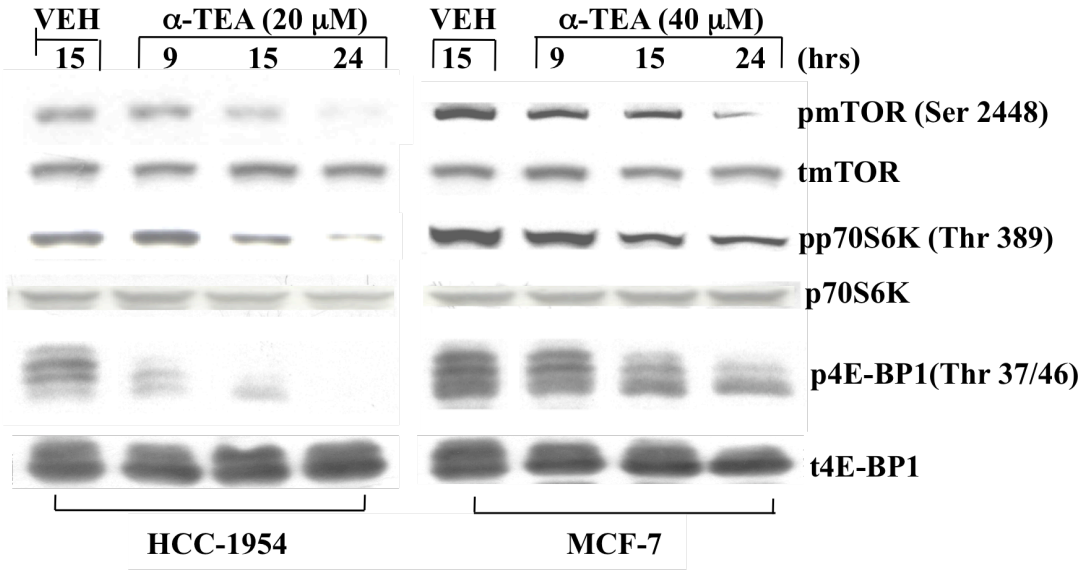


Figure 19. α-TEA suppresses AKT, ERK and mTOR as well as their downstream targets. HCC-1954 and MCF-7 cells were treated with 20 and 40 μM α-TEA, respectively, for 9, 15 and 24 hrs. (A) Protein levels of pAKT (Ser-473) and (Ser-308), pGSK (α/β) (Ser 21/9), pBad (Ser-136), pcaspase-9 (Ser-196) as well as levels of total AKT, GSK, Bad and caspase-9 were determined by western blot analyses. (B) Protein levels of pERK1/2, pp90RSK (Ser-380), and pBad (Ser-112) as well as total ERK, p90RSK and Bad were determined by western blot analyses. (C) Protein levels of pmTOR (Ser-2448), pp70S6K (Thr-389), and p4E-BP1 (Thr-37/46) and levels of total mTOR, p70S6K and 4E-BP1 were determined by western blot analyses. Data are representative of two separate experiments.

α-TEA reduces high basal levels of phosphorylated AKT, ERK1/2 and mTOR

VEH treated HCC-1954 and MCF-7 cells express high levels of active AKT (pAKT) and phosphorylated down-stream substrates GSK-3 (pGSK-3), Bad (pBad) and caspase 9 (pcaspase-9). α-TEA treatment of HCC-1954 (20 μM) and MCF-7 cells (40 μM) for 9, 15 and 24 hrs: (i) reduces levels of pAKT (both Ser-473 and Ser-308) as well as its downstream substrates pGSK-3 (α/β) (Ser 21/9), pBad (Ser-136) and pcaspase-9 (Ser-196) and increases cleaved caspase 9 in a time dependent manner (Fig 19 A), (ii) downregulates pERK1/2 and its substrates pp90SK (Ser-380) and pBad (Ser-112) (Fig 19 B), and (iii) downregulates pmTOR (Ser-2448) and its substrates pp70S6K (Thr-389) and p4E-BP1 (Thr-37/46) (Fig 19 C). These data show that α-TEA suppresses the active forms of AKT, ERK and mTOR and their downstream substrates, resulting in activation of pro-apoptotic mediators Bad and caspase-9.

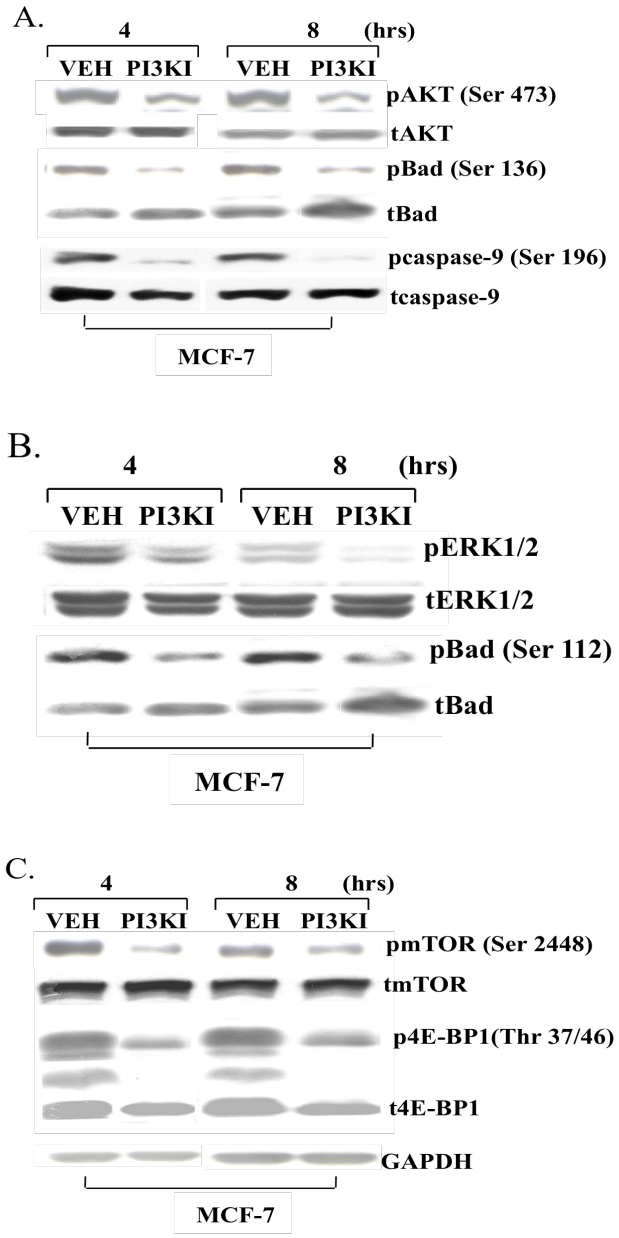
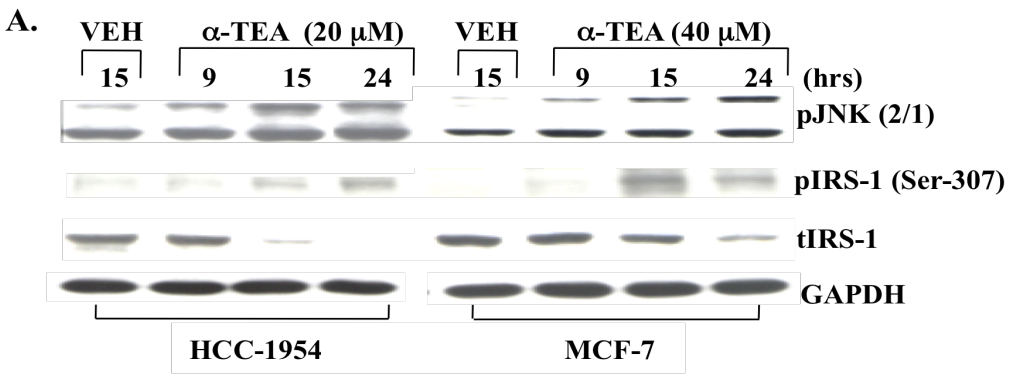
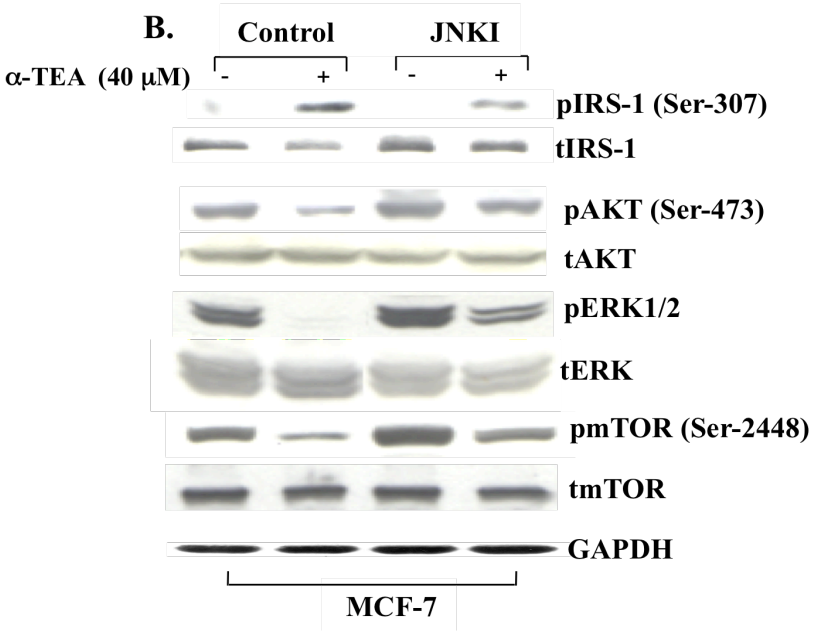


Figure 20. ERK and mTOR are downstream targets of PI3K. MCF-7 cells were treated with 1 μM PI3K inhibitor wortmannin for 4 and 8 hrs. (A) Protein levels of pAKT (Ser-473), pBad (Ser-136) and pcaspase-9 (Ser-196), and levels of total AKT, Bad and caspase-9 were determined by western blot analyses. (B) Protein levels of pERK1/2 and pBad (Ser-112) and total levels of ERK1/2 and Bad were determined by western blot analyses. (C) Protein levels of pmTOR (Ser 2448) and p4E-BP1 (Thr-37/46), and total levels of mTOR and 4E-BP1 were determined by western blot analyses. Data are representative of two separate experiments.

PI3K inhibitor (wortmannin) suppresses pAKT, pERK1/2 and pmTOR in MCF-7 cells

To better understand how the basal levels of pAKT, pERK and pmTOR are regulated, MCF-7 cells were treated with 1 μM wortmannin (PI3KI) for 4 and 8 hrs. Wortmannin reduced the phosphorylated forms of: (i) AKT and its substrates pBad (Ser-136) and pcaspase-9 (Ser-196) (Fig 20A), (ii) ERK1/2 and its substrate pBad (Ser-112) (Fig 20B), and pmTOR (Ser 2448) and its substrate p4E-BP1 (Thr 37/46) (Fig 20C), indicating that PI3K is a major contributor to the basal levels of AKT, ERK and mTOR.


A.
VEH
α-TEA (20 μM)
VEH
α-TEA (40 μM)
15
9
15
24
15
9
15
24
(hrs)
pJNK (2/1)
pIRS-1 (Ser-307)
tIRS-1
GAPDH
HCC-1954
MCF-7


B.
Control
JNKI
α-TEA (40 μM)
-
+
-
+
pIRS-1 (Ser-307)
tIRS-1
pAKT (Ser-473)
tAKT
pERK1/2
tERK
pmTOR (Ser-2448)
tmTOR
GAPDH
MCF-7

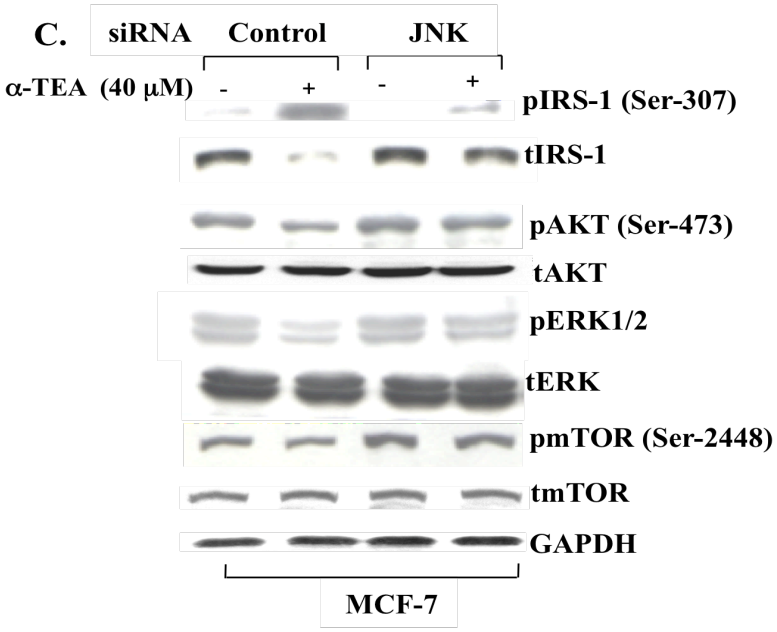
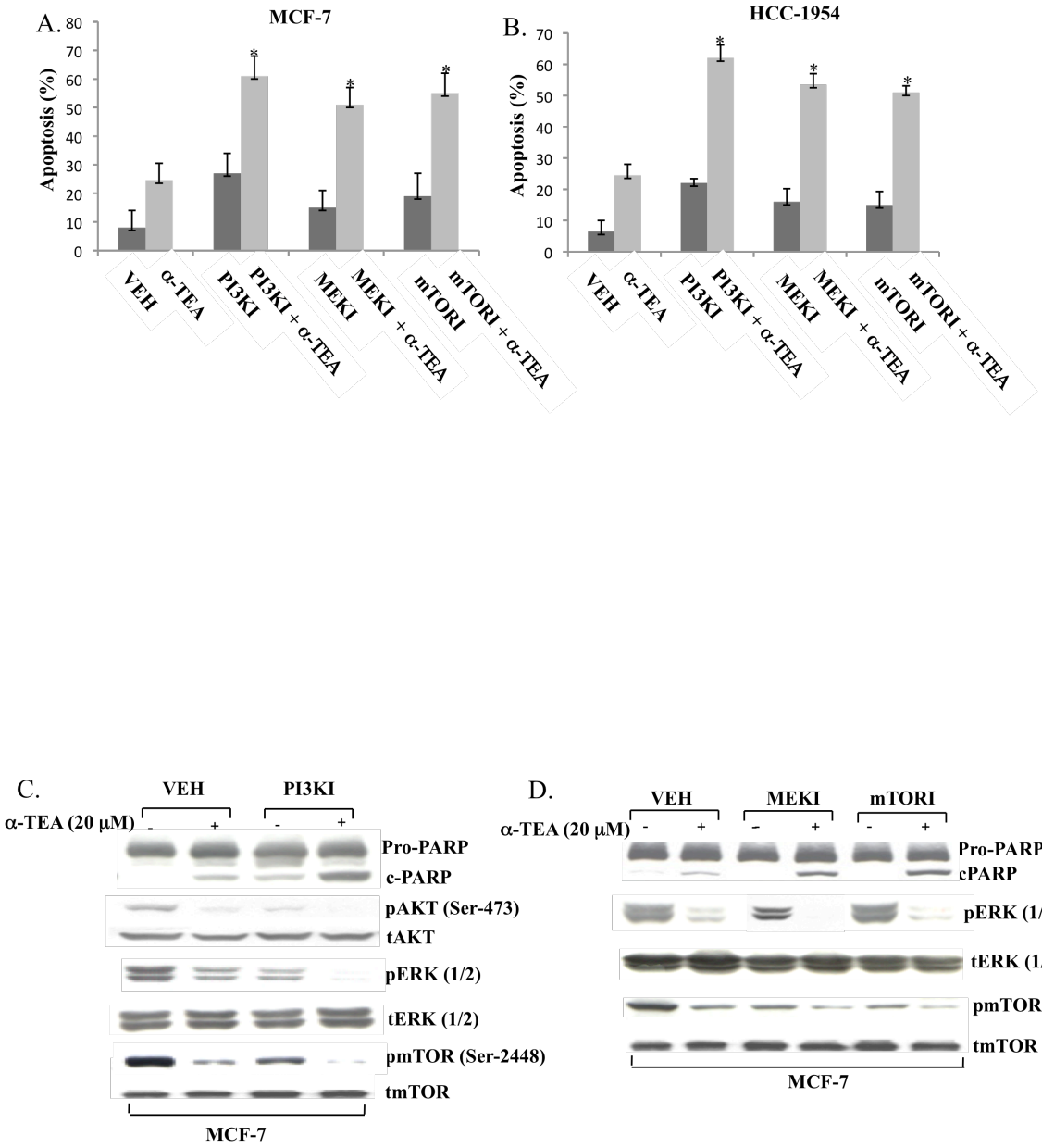
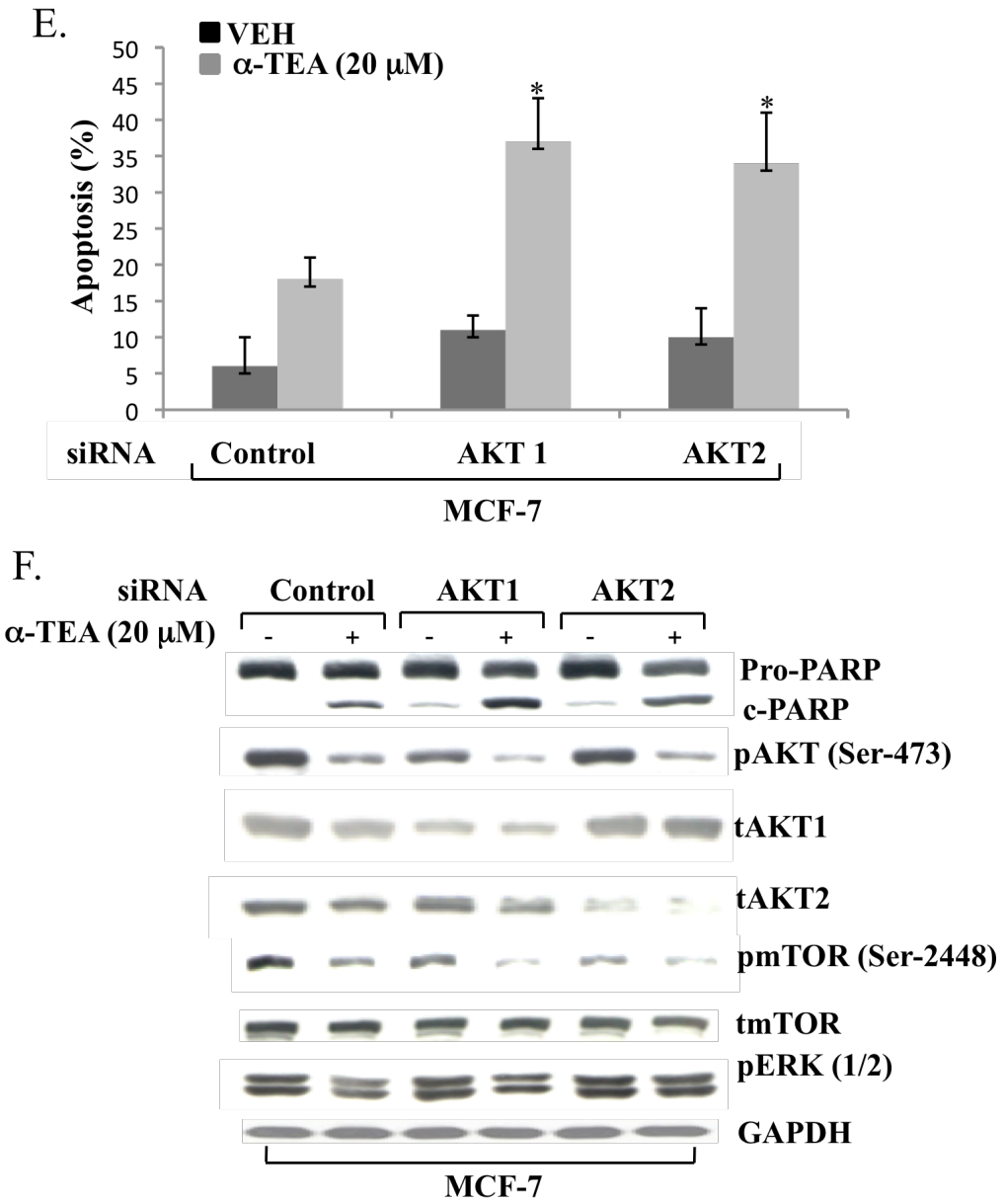


Figure 21. α-TEA downregulates AKT, ERK and mTOR via JNK mediated decrease in IRS protein expression. (A) HCC-1954 and MCF-7 cells were treated with 20 and 40 μM α-TEA, respectively, for 9, 15 and 24 hrs. Western blot analyses were performed to detect phospho-JNK2/1, pIRS-1 (Ser-307) and total IRS with GAPDH serving as loading control. (B) MCF-7 cells were treated with 10 μM JNK inhibitor SP600125 (JNKI) + 40 μM α-TEA for 18 hrs. Protein levels of pAKT (Ser-473), pERK1/2, pmTOR (Ser-2448), pIRS-1 (Ser-307) and total levels of AKT, ERK1/2, mTOR and IRS-1 were determined by western blot analyses with GAPDH serving as lane control. (C) MCF-7 cells transfected with siRNA to JNK or control siRNA were treated with 40 μM α-TEA for 18 hrs. Protein levels of pAKT (Ser-473), pERK1/2, pmTOR (Ser-2448), pIRS-1 (Ser-307) and levels of total AKT, ERK, mTOR, and IRS were determined by western blot analyses with GAPDH as lane control. Data are representative of two separate experiments.

α-TEA induces increased levels of pIRS-1 (Ser-307) and decreased levels of total IRS-1 via JNK

To identify a common up-stream factor that might account for how α-TEA suppresses pAKT, pERK and pmTOR in unison, the effect of α-TEA on pIRS-1 (Ser-307) was examined. Ser-307 is a phosphorylation site regulated by JNK for inactivation of IRS (Mamay et al, 2003) via reduction of total IRS protein levels (Hiratani et al, 2005). Since α-TEA has been reported to induce a prolonged activation of JNK (Yu et al, 2006; Shun et al, 2004; Jia et al, 2008; Wang et al, 2008), IRS-1 maybe a promising upstream target for α-TEA mediated events. HCC-1954 and MCF7 cells were treated with 20 and 40 μM α-TEA, respectively for 9, 15, and 24 hrs. α-TEA induced increased levels of pJNK2/1, increased levels of pIRS-1 (ser-307) and reduced levels of total IRS-1 in both cell lines (Fig 21A). Knockdown of JNK using a chemical inhibitor SP600125 (JNKI) (Fig 21B) or siRNA (Fig 21C) blocked α-TEA's ability to increase pIRS-1 (Ser-307) and decrease levels of total IRS-1 protein, and suppressed the ability of α-TEA to reduce pAKT (Ser-473), pERK1/2 and pmTOR (Ser-2448). These data suggest that downregulation of IRS-1 is involved in α-TEA's ability to suppress AKT, ERK and mTOR signaling and demonstrated that JNK is involved in this event via phosphorylation of IRS-1 at Ser-307, leading to its degradation.


A.
MCF-7
Apoptosis (%)
0
10
20
30
40
50
60
70
80
VEH
α-TEA
PI3KI
PI3KI + α-TEA
MEKI
MEKI + α-TEA
mTORI
mTORI + α-TEA
B.
HCC-1954
Apoptosis (%)
0
10
20
30
40
50
60
70
VEH
α-TEA
PI3KI
PI3KI + α-TEA
MEKI
MEKI + α-TEA
mTORI
mTORI + α-TEA
C.
VEH
PI3KI
α-TEA (20 μM)
- + - +
Pro-PARP
c-PARP
pAKT (Ser-473)
tAKT
pERK (1/2)
tERK (1/2)
pmTOR (Ser-2448)
tmTOR
MCF-7
D.
VEH
MEKI
mTORI
α-TEA (20 μM)
- + - + - +
Pro-PARP
cPARP
pERK (1/2)
tERK (1/2)
pmTOR (Ser-2448)
tmTOR
MCF-7


E.
VEH
α-TEA (20 μM)
Apoptosis (%)
50
45
40
35
30
25
20
15
10
5
0
*
*
siRNA
Control
AKT 1
AKT2
MCF-7
F.
siRNA
Control
AKT1
AKT2
α-TEA (20 μM)
- + - + - +
Pro-PARP
c-PARP
pAKT (Ser-473)
tAKT1
tAKT2
pmTOR (Ser-2448)
tmTOR
pERK (1/2)
GAPDH
MCF-7

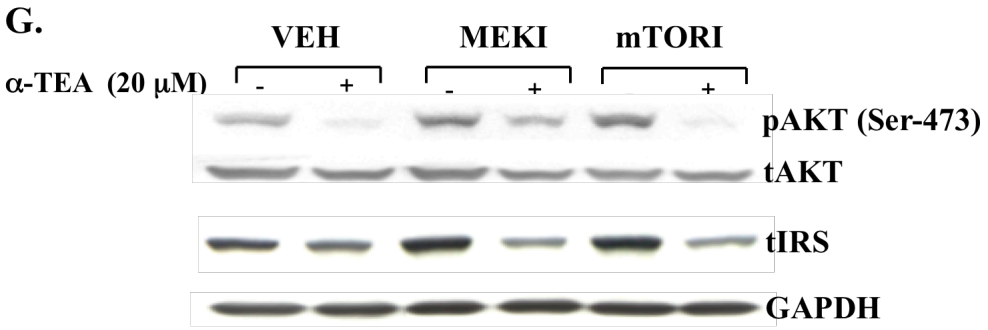


Figure 22. Inhibitors of PI3K, MEK/ERK and mTOR, as well as siRNAs to AKT1 and AKT2 enhance α-TEA-induced apoptosis. (A and B). HCC-1954 and MCF-7 cells were treated with 1 μM PI3K inhibitor wortmannin (PI3KI), 10 μM MEK inhibitor U01260 (MEKI) or 50 nM mTOR inhibitor rapamycin (mTORI) plus10 μM and 20 μM of α-TEA for 18 hrs, respectively. Apoptosis was determined by Annexin V/PI staining. (C and D). Protein levels of cleaved PARP, pAKT (Ser-473), pERK1/2 and pmTOR (Ser-2448) as well as levels of total AKT, ERK1/2 and mTOR were determined by western blot analyses in MCF-7 cells treated with the three inhibitors plus α-TEA. (E) MCF-7 cells transfected with siRNAs to AKT1, AKT2 or control was treated with 20 μM α-TEA for 18 hrs. Apoptosis was determined by Annexin V/PI staining. (F) Protein levels of cleaved PARP, pAKT (Ser-473), pmTOR (Ser-2448), pERK1/2, and levels of total AKT1, AKT2, and mTOR were determined by western blot analyses. Levels of GAPDH served as lane controls. (G) MCF-7 cells treated with 10 μM MEK inhibitor U01260 (MEKI) or 50 nM mTOR inhibitor rapamycin (mTORI) plus 20 μM of α-TEA for 18 hrs. Protein levels of pAKT (Ser-473), AKT, IRS-1 and GAPDH were determined by western blot analyses. Data in C, D, F and G are representative of two separate experiments. Data in A, B and E are presented as mean $\pm$ SD of three independent experiments. * = Significantly different from control (p<0.05).

Inhibitors of PI3K, MEK/ERK and mTOR as well as siRNA to AKT1 and AKT2 cooperated with α-TEA to induce apoptosis.

To determine the effects of inhibition of members of the PI3K pathway on α-TEA induced apoptosis, HCC-1954 and MCF-7 cells were cultured with 10 and 20 μM α-TEA, respectively, plus 1 μM PI3K inhibitor (PI3KI) wortmannin, 10 μM MEK inhibitor (MEKI) U01260 and 50 nM mTOR inhibitor (mTORI) rapamycin for 18 hours. Single treatments with α-TEA or inhibitors induced low levels of apoptosis, while combinations of α-TEA plus each of the three inhibitors individually significantly enhanced induction of apoptosis in both cell types in comparison with single treatments (Fig 22 A & B). Both α-TEA and PI3K inhibitor reduced levels of pAKT (Ser-473), pERK1/2, and pmTOR (Ser 2448) and the combination of α-TEA + PI3K inhibitor acted cooperatively to enhance PARP cleavage and to reduce levels of pAKT (Ser-473), pERK1/2, and pmTOR (Ser-2448) when compared with individual treatments or control (Fig 22 C). Both MEK and mTOR inhibitors enhanced α-TEA induced PARP cleavage and cooperatively enhanced α-TEA inhibition of pERK1/2 and pmTOR, respectively (Fig 22 D). Combinations of α-TEA + AKT1 or 2 siRNA acted cooperatively to induce apoptosis (Fig 22 E) and inhibit pAKT (Ser-473) and total AKT1 and AKT2, respectively, as well as pmTOR (Ser 2448), but had no effect on basal levels of pERK1/2, showing that the ERK signaling pathway is independent of

AKT signaling (Fig 22 F). Taken together, data in Figure 5 show that α-TEA can down regulate the basal levels of active AKT, ERK1/2 and mTOR and can act cooperatively with either chemical or genetic-based inhibitors of these pro-survival mediators to enhance breast cancer cell death.

α-TEA blocked MEK and mTOR inhibitor's ability to induce increase levels of pAkt and IRS

It has been reported that MEK and mTOR inhibitors induce increased levels of phospho-Akt via negative feedback regulation of IRS (Wan et al, 2007; Jiang et al, 2009). As expected, MEK and mTOR inhibitors induced increased levels of pAKT and IRS-1, an outcome that limits their clinical anticancer efficacy; and importantly co-treatment with α-TEA was able to block this counter productive increase in these potent pro-survival mediators (Fig 22 G).

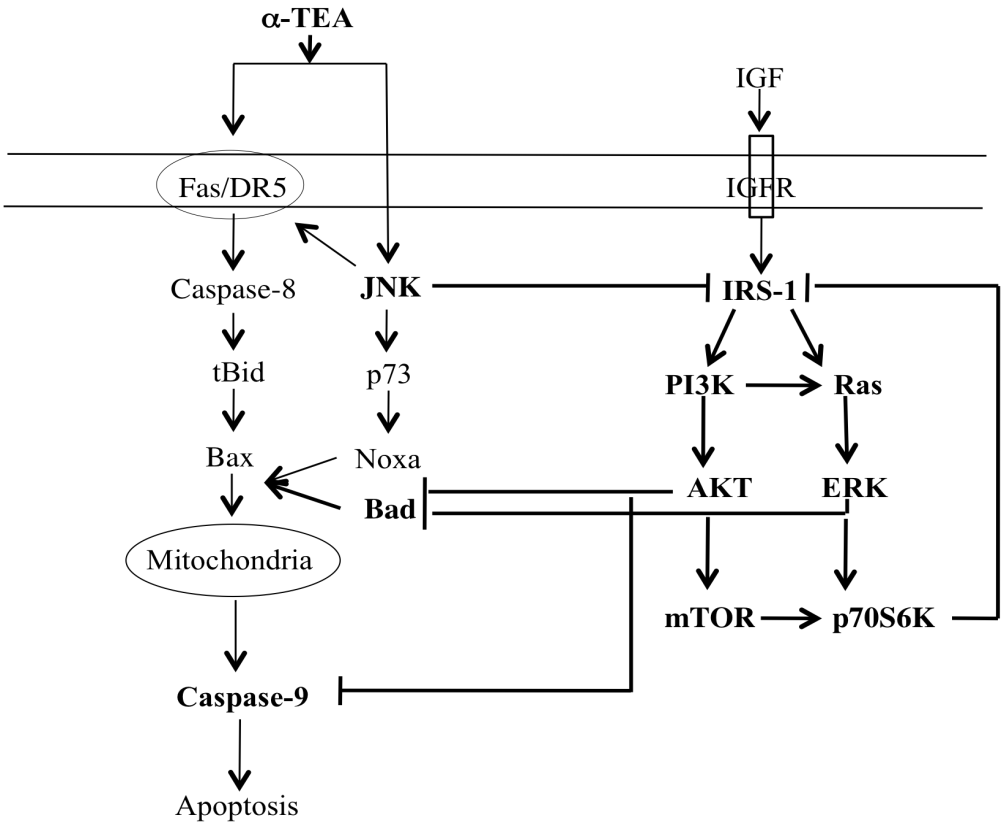


Figure 23. Schematic diagram depicting signaling pathways that are involved in α-TEA alone and α-TEA + MEK or mTOR inhibitors induced apoptosis. Schematic diagram depicts cross-talk between survival and death signaling pathways in α-TEA alone and α-TEA + MEK or mTOR inhibitor induced apoptosis in human breast cancer cells. Constitutively active Insulin Receptor Substrate-1 (IRS-1) activates AKT and ERK via phosphorylations mediated by PI3K and Ras respectively, leading to subsequent phosphorylation events that inhibit pro-apoptotic mediators Bad and Caspase 9, and activate pro-survival mTOR. Beneficial antitumor actions by inhibitors of mTOR (Rapamycin) and ERK (MEK inhibitor) are compromised by elimination of mTOR or ERK regulated p70S6K negative loop inhibiting IRS-1 signaling. The loss of this negative feedback loop results in continued activation of AKT signaling and inhibition of apoptosis by inactivation of Bad and Caspase 9. α-TEA induces death receptor mediated apoptosis (depicted by non-bolded signaling pathways described in references (Yu et al, 2006; Shun et al, 2004; Jia et al, 2008; Wang et al, 2008) with involvement of prolonged activation of JNK. Active JNK blocks IRS-1 signaling, thereby, down-regulating downstream survival signaling mediators, resulting in the activation of pro-apoptotic mediators Bad and caspase 9, leading to promoting mitochondria-dependent apoptosis. Combinations of sub-optimal levels of α-TEA plus mTOR or MEK inhibitors act synergistically to induce apoptosis by reduction of AKT- and ERK-mediated inhibition of pro-apoptotic factors Bad and caspase 9.

Based on these data, we hypothesize a signaling pathway depicting cross-talk between survival and death signaling pathways in α-TEA alone and α-TEA + MEK or mTOR inhibitor induced apoptosis in human breast cancer cells (Fig 23). In the untreated cells, IGF/IGFR via downstream mediator IRS-1 regulates two distinct signaling pathways (PI3K and Ras) that contribute to basal levels of activated AKT, ERK, and mTOR. Activated AKT and ERK1/2 phosphorylate pro-apoptotic mediators Bad and caspase-9 thereby inhibiting their pro-apoptotic actions thus enhancing cancer cell survival. ERK and mTOR trigger a negative feed-back loop via their substrate p70S6K that downregulates IRS-1 signaling. Previous studies (non-bolded signaling pathway) have shown that α-TEA induces apoptosis via up-regulation of cell surface death receptor mediated caspase-8 and mitochondrial-dependent pathways (Yu et al, 2006; Shun et al, 2004; Jia et al, 2008; unpublished data). Previous studies have also identified c-Jun N-terminal kinase (JNK) as a key player in α-TEA induced apoptosis (Yu et al, 2006; Shun et al, 2004; Jia et al, 2008; Wang et al, 2008). In addition to signaling apoptosis via p73/Noxa (Wang et al, 2008) and a Fas/JNK positive feedback loop (Jia et al, 2008), here we report that JNK suppresses PI3K pro-survival signaling pathways via phosphorylation (inactivation and degradation) of IRS-1. Since IRS-1 plays an apical role in activation of AKT, ERK, and mTOR survival signaling pathways via PI3K, elimination of IRS-1 deprives the cells of these signaling mediators as well as restores pro-apoptotic activity by Bad and caspase-9 via their

dephosphorylation leading to cell death by apoptosis. In summary, results reported here help clarify why α-TEA is such a potent apoptotic agent in human breast cancer cell lines, and suggest potential utility for α-TEA in combination therapy with ERK and mTOR inhibitors.

3.4. Discussion

These studies demonstrate, for the first time, that α-TEA suppresses AKT and ERK mediated anti-apoptotic events; namely, inhibitory phosphorylation of two pro-apoptotic factors Bad and caspase-9. Additionally, α-TEA suppression of AKT leads to decreases in mTOR activity as measured by reduction in downstream mediators p4E-BP1 and p70S6K. Functional knockdown assessments indicate that α-TEA mediated activation of JNK plays a critical role in these events via downregulation of IRS-1. The following sequence of events are proposed for α-TEA mediated apoptosis: α-TEA inhibits PI3K via JNK mediated phosphorylation of IRS-1 at ser-307, resulting in inactivation of AKT/mTOR and Ras/ERK, which acts cooperatively in α-TEA induced mitochondrial-dependent pro-apoptotic events via activation of Bad and caspase-9. Of special note, these studies showed that the harmful outcome of drug inhibition of either ERK or mTOR; namely, activation of AKT, could be prevented by combination treatment with α-TEA. In summary, data show that α-TEA suppression of IRS-1 not only plays an important role in α-TEA induced apoptosis but also can suppress activation of AKT induced by ERK and mTOR inhibitors, suggesting that α-TEA might improve clinical outcomes of treatment with ERK or mTOR inhibitors.

Pleiotropic effects of PI3K/AKT signaling on inhibition of apoptosis have been reported to be a major mechanism for drug resistance (Falasca, 2010; Gibbons et

al, 2009; McCubrey et al, 2007). Therefore, identification of agents that can both block survival and induce death signaling pathways should aid development of strategies to sensitize drug resistant breast cancer cells. α-TEA has been reported to suppress AKT in prostate and ovarian cancer cells and that suppression of AKT contributes to α-TEA induced apoptosis (Jia et al, 2008; Yu et al, 2006; Shun et al, 2010). In prostate cancer cells, α-TEA suppression of AKT causes activation of Fox-1, a pro-apoptotic transcription factor capable of triggering apoptosis via upregulation of Bim (Jia et al, 2008). In ovarian cancer cells, α-TEA suppresses c-FLIP and survivin protein expression via AKT-mediated events (Shun et al, 2010). Here, we report that α-TEA suppression of AKT via targeting IRS-1/PI3K, leads to activation of pro-apoptotic Bad and caspase-9 in human breast cancer cells.

Bad is a pro-apoptotic member of the Bcl-2 family, which belongs to the BH3-only protein family, comprised of Bad, Bik, Bmf, Hrk, Noxa, tBid, Bim and Puma (Kim et al, 2006). Bad (Bcl-2 associated death promoter, Bcl-2 antagonist of cell death) is a pro-apoptotic factor that promotes apoptosis via forming heterodimers with pro-survival proteins Bcl-2 and Bcl-xL, preventing them from binding with Bax (Yang et al, 1995). Phosphorylation of Bad at Ser-112 and Ser-136, disrupts Bad association with Bcl-2 or Bcl-xL, promoting cell survival (Datta et al, 2000; Hayakawa et al, 2000). Phosphorylation of Bad at ser-112 and ser-136 has been proposed to be mediated by ERK/p90RSK1 and PI3K/AKT pathways,

respectively (Hayakawa et al, 2000). Thus, the phosphorylation status of Bad at these critical serine residues serves as a determinant of either cell death or survival (Downward, 1999). Data showing that α-TEA reduces phosphorylation of Bad at both ser-112 and ser-136 sites, suggest that α-TEA's ability to reduce AKT and ERK activities contributes to restoration of Bad's pro-apoptotic function. Furthermore, data showing that inhibition of PI3K with wortmannin suppressed phosphorylation of Bad at both ser-112 and ser-136, supports a role for PI3K in regulating both AKT and ERK/p90RSK1 in these cells. Previous data from our lab showed that α-TEA induces mitochondria-dependent apoptosis via activation of Bax, a critical step in triggering mitochondria-dependent apoptosis (Jia et al, 2008; Yu et al, 2006; unpublished data). Two other BH3 only pro-apoptotic Bcl-2 members; caspase-8-mediated tBid (active form of Bid) and p73-mediated Noxa have been shown to be upregulated by α-TEA (Jia et al, 2008; Yu et al, 2006; Wang et al, 2008; unpublished data). Here, for the first time, we report that α-TEA induces Bad activation via inhibiting AKT and ERK. These data demonstrate that α-TEA triggers mitochondria-dependent apoptosis via targeting different Bcl-2-associated death promoters, namely, Bid, Noxa and Bad in cancer cell types.

Caspase-9, a mitochondria-mediated initiation caspase, is directly activated by Apaf-1 and cytochrome c and triggers activation of execution caspases 3, 6 and 7, leading to DNA fragmentation and cell death (Li et al, 1997). It has been reported

that caspase-9 activity is regulated by phosphorylation (Cardone et al, 1998). AKT phosphorylates caspase-9 at Ser-196 leading to inactivation of caspase-9 (Cardone et al, 1998). Therefore, caspase-9 is another target for AKT to prevent cells from undergoing apoptosis. Thus, α-TEA suppression of AKT phosphorylation of caspase-9 at ser-196 contributes to α-TEA-induced mitochondria-dependent apoptosis.

mTOR is a downstream mediator of PI3K/AKT signaling, regulating proliferation, survival, mobility and angiogenesis via targeting p70S6 kinase (p70S6K) and 4E-BP1 in breast cancers that exhibit constitutively activated PI3K/AKT signaling (Bjornsti et al, 2004). Accumulating evidence suggests that PI3K/AKT/mTOR promotes breast cancer cell survival and resistance to chemotherapetics such as trastuzumab (a blocking antibody to Her-2) and tamoxifen (Hynes et al, 2009; Ghayad et al, 2010). mTOR inhibitors rapamycin and rapamycin analogs (CCI-779, RAD001, AP23573) have exhibited impressive growth inhibitory effects against a broad range of human cancers, including breast cancer, in preclinical and early clinical studies (Chan, 2004; Vignot et al, 2005). Here, we demonstrate that α-TEA functions as an mTOR inhibitor, capable of suppressing mTOR by decreasing constitutively activated mTOR (phosphorylated status of mTOR) and its downstream mediators p70S6K and 4E-BP1. In addition, our data show that α-TEA not only enhances rapamycin suppression of mTOR and induction of apoptosis, but also suppresses rapamycin-mediated feedback

activation of AKT, providing a rationale for developing a combination regimen of mTOR + α-TEA for breast cancer treatment.

IRS-1 (Insulin Receptor Substrate-1) is an adaptor protein important for the insulin receptor and IGF-1 receptor (Insulin-like Growth Factor-1 Receptor) signal transduction to downstream targets, including PI3K (Valentinis et al, 2001; Surmacz, 2000). IRS-1 plays important roles in maintaining insulin sensitivity in adipocytes and cell growth in cancer cells (Hartley et al, 2002). IRS-1 activity is positively and negatively regulated via phosphorylation of IRS-1 at different sites by not only ligand activated cell surface receptors but also by different intracellular Ser/Thr protein kinases, including mammalian target of rapamycin (mTOR), ERK, protein kinase C, and AMP-activated protein kinase, as well as JNK (Hiratani et al, 2005; Mingo-Sion et al, 2005; Rui et al, 2001; Horike et al, 2003; Ozes et al, 2001; De Fea et al, 1997). IRS-1 Ser-307 lies near the phospho-tyrosine binding domain in IRS-1 and confers an inhibitory effect on both insulin and IGF-1 signaling (Greene et al, 2003). Activation of JNK has been established as a stress-mediated inducer of insulin resistance in diabetic animal models via phosphorylation of IRS-1 at Ser-307, leading to inactivation of IRS-1 by interfering with the interaction of the insulin receptor and IRS-1 and promoting IRS-1 degradation (Mamay et al, 2003). An inhibitory effect of JNK on IRS-1 activity via phosphorylation at ser-307 in human breast cancer cells has also been reported in Taxol treatments (Mamay et al, 2003). Here, we report that α-TEA

functions as an IRS-1 suppressor in human breast cancer cells via JNK-dependent phosphorylation of IRS-1 at ser-307. α-TEA mediated phosphorylation of IRS-1 at ser-307 is correlated with downregulation of total protein levels of IRS-1 and both α-TEA mediated phosphorylation of IRS-1 and downregulation of total protein level of IRS-1 are JNK-dependent, suggesting that α-TEA downregulation of total protein level of IRS-1 may be subsequent to phosphorylation of IRS-1 at ser-307 since phosphorylation of IRS-1 at ser-307 has been reported to decrease IRS-1 stability (Greene et al, 2003). mTOR and ERK have been reported to negatively regulate IRS-1 via their downstream mediator p70S6K (Wan et al, 2007; Jiang et al, 2009), providing a negative feedback mechanism for turning off activation of AKT (Sun et al, 2005). Thus, inhibitors of MEK/ERK and mTOR produce a counter productive increase in pAKT via loss of the negative feedback control of IRS-1 levels, which limits their clinic applications. It is highly significant that α-TEA functions not only as an inhibitor of ERK and mTOR, but also as an inhibitor of AKT. It is also highly significant that α-TEA in combination with ERK and mTOR inhibitors blocks inhibitor-induced increases in pAKT. These potent anticancer actions by α-TEA appears to be due to α-TEA's ability to inhibit IRS-1/PI3K via activation of JNK.

JNK has been reported to be involved in α-TEA-induced Fas and Fas L protein expression in prostate cancer (Jia et al, 2008), as well as p73/Noxa protein expression (Wang et al, 2008) in breast cancer cells. Here, we report that JNK, a

pro-apoptotic mediator suppressed pro-survival PI3K and its downstream mediators via targeting IRS-1. These data demonstrate that JNK plays a critical pleiotropic role in α-TEA-induced apoptosis, both pro-apoptotic and anti-survival.

In summary, α-TEA's ability to target PI3K mediated pro-survival factors via JNK mediated inhibition of IRS-1 not only plays a role in enhancing α-TEA induced apoptosis by inhibition of AKT, ERK and mTOR and their downstream mediators, but also suggests a novel means for preventing pro-cancer impact of AKT activation induced by ERK and mTOR inhibitors.

Chapter 4. α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53 mutant, triple negative human breast cancer cells via activating p73

4.1. Introduction

Successful treatment of triple negative (estrogen receptor negative, progesterone receptor negative and Her-2 negative) breast cancers (TNBC) that are also p53 mutant remains elusive. Unfortunately, the anticancer efficacy of commonly used chemotherapeutic agents for TNBC, including doxorubicin (DOXO) and cisplatin (CDDP), are limited due to acquired drug resistance and toxicities [Oakman et al., 2010; Isakoff et al., 2010].

DOXO and CDDP are DNA damaging drugs that exert their anticancer actions via inhibition of cellular proliferation and induction of cell death by apoptosis [Yoshida et al., 2010; Flores et al., 2002]. Tumor suppressor gene p53 plays a central role in the anticancer actions of DNA damaging agents. Loss of wild-type p53 functions leads to resistance to DNA damaging agents, such as DOXO and CDDP [Aas et al., 1996; Branch et al., 2000]. Identification of anticancer agents that target p53 downstream genes via p53-independent mechanisms is of major clinical relevance, especially since p53 deficiency is a hallmark of many different cancer types.

p73 is a member of the p53 gene family [Jost et al., 1997]. Unlike p53, p73 is rarely mutated or lost in cancers [Hainaut et al., 1997]. Although p53 deficient cancers are less responsive to chemotherapy, they are typically not completely drug resistant because other p53 family members such as p73, can replace p53 function in response to DNA damage [Vayssade et al., 2005; Irwin et al., 2003]. Since p73 is usually not mutated in cancers and has been shown to regulate p53 target genes in p53 deficient cancers, identification of anti-cancer agents that can activate p73 in p53-deficient cancers will provide a chemotherapeutic approach for treatment of drug resistant p53 mutant cancers.

α-TEA (2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl) chroman-6-yloxyacetic acid, called RRR-α-tocopherol ether-linked acetic acid analog or RRR-α-tocopheryloxyacetic acid, is a non-hydrolyzable ether analog of RRR-α-tocopherol [Lawson et al., 2003]. α-TEA has been shown to be a potent pro-apoptotic agent both in *vitro* and *in vivo* in breast, prostate and ovarian cancer cells [Anderson et al., 2004; Lawson et al., 2004; Hahn et al., 2006; Jia et al., 2008; Yu et al., 2006; Kline et al., 2007]. Recently, α-TEA has been shown to delay tumor onset and inhibit the progression and metastatic spread in a clinically relevant model of spontaneous mammary cancer, further highlighting the translational potential of this anticancer agent [Hahn et al., 2009]. Mechanisms involved in α-TEA induced apoptosis include: activation of JNK/c-Jun, p73/NOXA and Fas/DR5 and suppression of c-FLIP-L, survivin and phospho-Akt

(pAkt), leading to death receptor mediated caspase-8 activation and mitochondria dependent apoptosis [Shun et al., 2004; Yu et al., 2006; Jia et al., 2008; Yu et al., 2009; Wang et al., 2008; Shun et al., 2010].

Data presented here show that α-TEA in combination with DOXO or CDDP significantly enhances apoptosis of p53 mutant, triple negative human breast cancer cells by targeting p73 mediated p53-dependent pro-apoptotic and anti-apoptotic genes via c-Abl, JNK and Yap signaling pathways.

4.2. Materials and Methods

Chemicals

α-TEA was made in house as previously described [Lawson et al., 2004]. DOXO and CDDP were purchased from Sigma (San Diego, CA). PI3K inhibitor (wortmannin) was purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

p53 mutant, triple negative human breast cancer cell lines MDA-MB-231, BT-20 and MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA). MDA-MB-231 and BT-20 cells were cultured in MEM media with 10% FBS and MDA-MB-468 cells were cultured in DMEM media with 10% FBS. All three p53 mutant TNBC cell lines (ER-, PR-, HER2 -/low) used in these studies were originally obtained from human samples and no exact isogenic controls expressing wildtype p53, estrogen receptor and progesterone receptor can be found for comparison purposes. For experiments, FBS was reduced to 2% to better mimic low *in vivo* serum exposure and cells were allowed to attach overnight before treatments. α-TEA (40 mM) was dissolved in ethanol as stock solution. Concentrations of ethanol used in vehicle (VEH) treatments were 0.025-0.05% (v/v) to match ethanol content in the different final concentrations of α-TEA treatments. DOXO and CDDP were dissolved in H_2O.

Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturer's instructions. 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 (i.e. positive for annexin-V fluorescence) were considered to be apoptotic.

Nuclear and cytoplasmic fractionation

Cytoplasmic and nuclear fractions were prepared as previously described [21]. Briefly, whole cell lysates were centrifuged to obtain supernatant and pellet. The supernatant was centrifuged again and the resulting supernatant was used as the cytosolic fraction. The pellet was layered over a cushion of 1 ml sucrose buffer and centrifuged. The final pellet, lysed using RIPA buffer was used as nuclei fraction.

Western blot analyses

Whole cell protein lysates were prepared and western blot analyses were conducted as described previously [22]. Proteins at 20-50 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), Fas, Bcl-2, Bax, total JNK and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA); p73

and NOXA (Imgenex, San-Diego, CA); pYap (Ser-127), Yap, p-cAbl (Tyr-245), c-Abl, pAkt (Ser-473), caspase-8, caspase-9, DR5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Beverly, MA).

RT-PCR detection of Fas, DR5, Bax, Noxa and Bcl-2 mRNA expression

Total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect Fas, DR5, Bax, Noxa and Bcl-2 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) using the housekeeping gene β-actin as control. Total RNA was reverse transcribed to cDNA using Superscript RTase (250 U, Invitrogen, Carlsbad, CA) following the manufacture's instructions. cDNA was used per PCR reaction with Taq PCR Master Mix Kit (Qiagen, Inc) plus 10 μM oligonucleotide primer pairs (Invitrogen).

RNA interference

A scrambled RNA duplex purchased from Ambion (Austin, TX) that does not target any known mouse, rat or human gene was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of MDA-MB-231 cells with siRNA to p73, c-Abl, JNK, Yap or control (Ambion) was performed in 100 mm cell culture dishes at a density of 2×10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After one day of exposure to

transfection mixture, the cells were re-cultured in 100 mm dish at 2 x 10^6 cells/dish and incubated for one day followed by treatments.

Statistical Analysis

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey test for comparison of more than two treatments or a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p<0.05$.

4.3. Results

α-TEA, DOXO and CDDP induce apoptosis in p53 mutant, triple negative human breast cancer cells

The sensitivity of three p53 mutant, TNBC lines (MDA-MB-231, BT-20 and MDA-MB-468) to apoptosis induced by α-TEA, DOXO and CDDP was evaluated by determining EC_{50} values for apoptosis (Table 1). Data show that MDA-MB-468 cells exhibit the most sensitive phenotype and MDA-MB-231 cells exhibit the most resistant phenotype to apoptosis induced by DOXO and CDDP among the three cell lines. While the sensitivity of the three cell lines to α-TEA induced apoptosis is similar.

Table 1. EC_{50} values for apoptosis [a,b]

Cell lines	α-TEA (μM)	DOXO (μM)	CDDP (μM)
MDA-MB-231	**41.7**	**46.5**	**70.8**
BT-20	**45.4**	**25.7**	**64.0**
MDA-MB-468	**35.4**	**8.5**	**40.7**

[a]Cells were treated with different concentrations of α-TEA, DOXO, and CDDP for 24 hrs. Apoptosis was determined by Annexin V-FITC/PI staining/FACS as described in Material and Methods.

[b]The concentration that achieved 50% apoptosis (half maximal effective concentration; EC_{50}) was determined using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).

α-TEA cooperates with DOXO and CDDP to induce apoptosis of p53 mutant TNBC cells

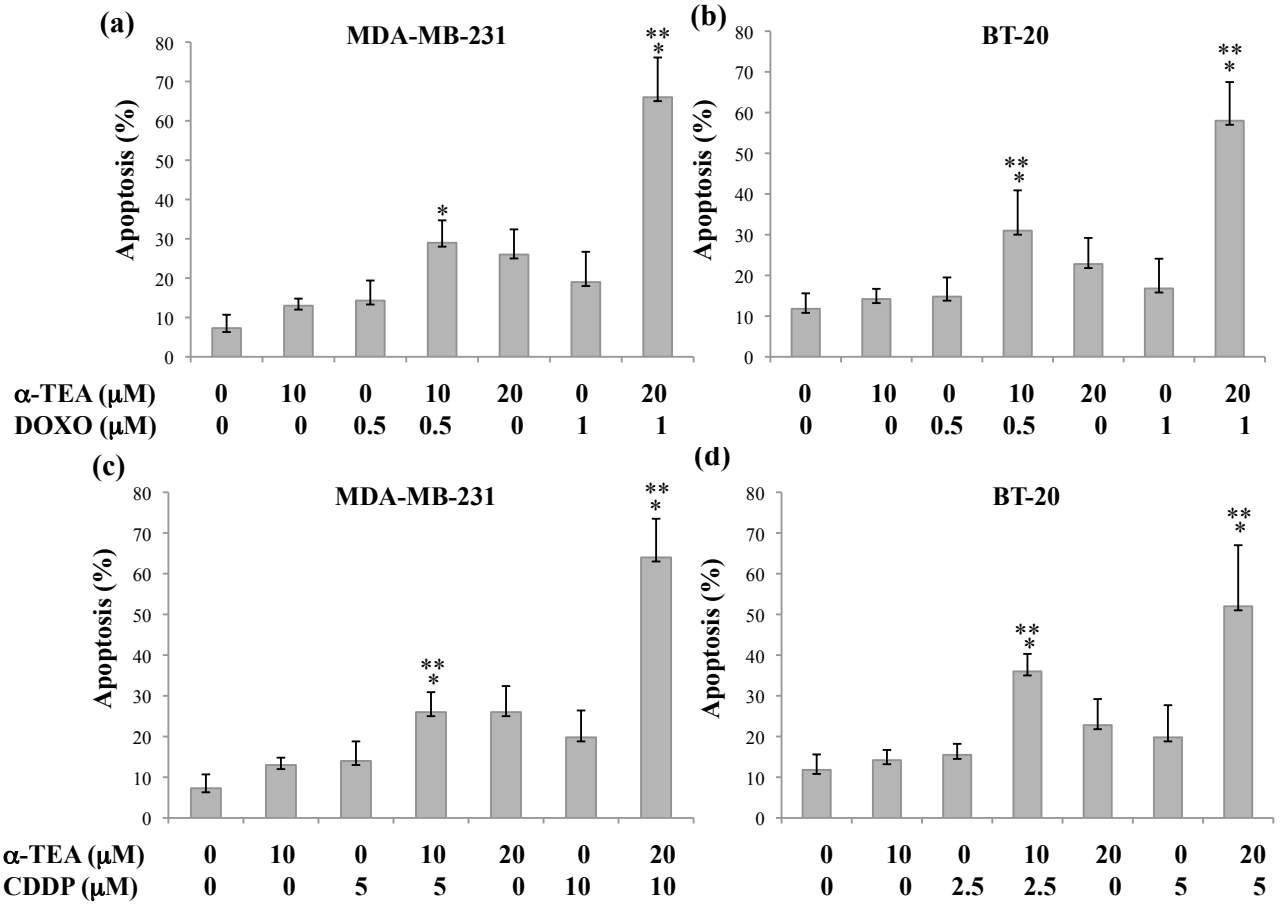


Figure 24. α-TEA cooperates with DOXO and CDDP to induce apoptosis of p53 mutant, triple negative human breast cancer cells. MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of DOXO or CDDP alone or in combination with α-TEA for 24 hrs. FACS/Annexin V assays were used to determine the percentage of apoptotic cells (a, b, c & d). Data are expressed as mean ± SD from three independent experiments. *p<0.05=significantly different from control and **p<0.05=significantly different from single treatments.

Based on the EC_{50} values for apoptosis presented in Table 2, MDA-MB-231 and BT-20 cell lines, which are more resistant to DOXO and CDDP were chosen to study the combinational effects of α-TEA + DOXO or CDDP on apoptosis induction. Data showed that α-TEA at 10 and 20 μM significantly enhanced apoptosis in combination with DOXO and CDDP in MDA-MB-231 and BT-20 cells, respectively, in comparison to individual treatments (Fig 24 A, B, C, and D). Mean CI (combination index) for combination of α-TEA + DOXO was 0.41 $\pm$ 0.07 and 0.53 $\pm$ 0.05 for MDA-MB-231 and BT-20 cells, respectively (Table 2). Mean CI for combination of α-TEA + CDDP was 0.45 $\pm$ 0.10 and 0.75 $\pm$ 0.08 in MDA-MB-231 and BT-20 cells, respectively (Table 2). These data demonstrate that combinations of α-TEA + DOXO or CDDP synergistically induce apoptosis in both cell lines. Western blot analyses show that α-TEA at 20 μM cooperates with DOXO and CDDP to induce elevated levels of cleaved caspases-8, -9, and PARP in both cell lines (Fig 25 A and B), indicating that apoptosis induced by these combinations involves both caspase-8 and caspase-9 activation.

Table 2 Combination Index (CI) of apoptosis [a]

	α-TEA:DOXO Ratio [b]	CI [c]			Mean ± SD [d]	
		ED50	ED75	ED90		
MDA-MB-231	20:1	0.48	0.40	0.35	0.41 ± 0.07	Synergism [e]
BT-20	20:1	0.48	0.52	0.58	0.53 ± 0.05	Synergism
	α-TEA:CDDP Ratio	CI			Mean ± SD	
		ED50	ED75	ED90		
MDA-MB-231	2:1	0.55	0.44	0.36	0.45 ± 0.10	Synergism
BT-20	4:1	0.68	0.74	0.84	0.75 ± 0.08	Synergism

[a]MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of α-TEA, DOXO and CDDP alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining /FACS assay as described in Material and Methods.

[b]The ratio for the concentrations that were used in combination treatments were determined from the data in Fig1.

[c]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).

[d]The mean ± SD is calculated from the CI values of ED50, ED75 and ED90.

[e]CI values < 1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

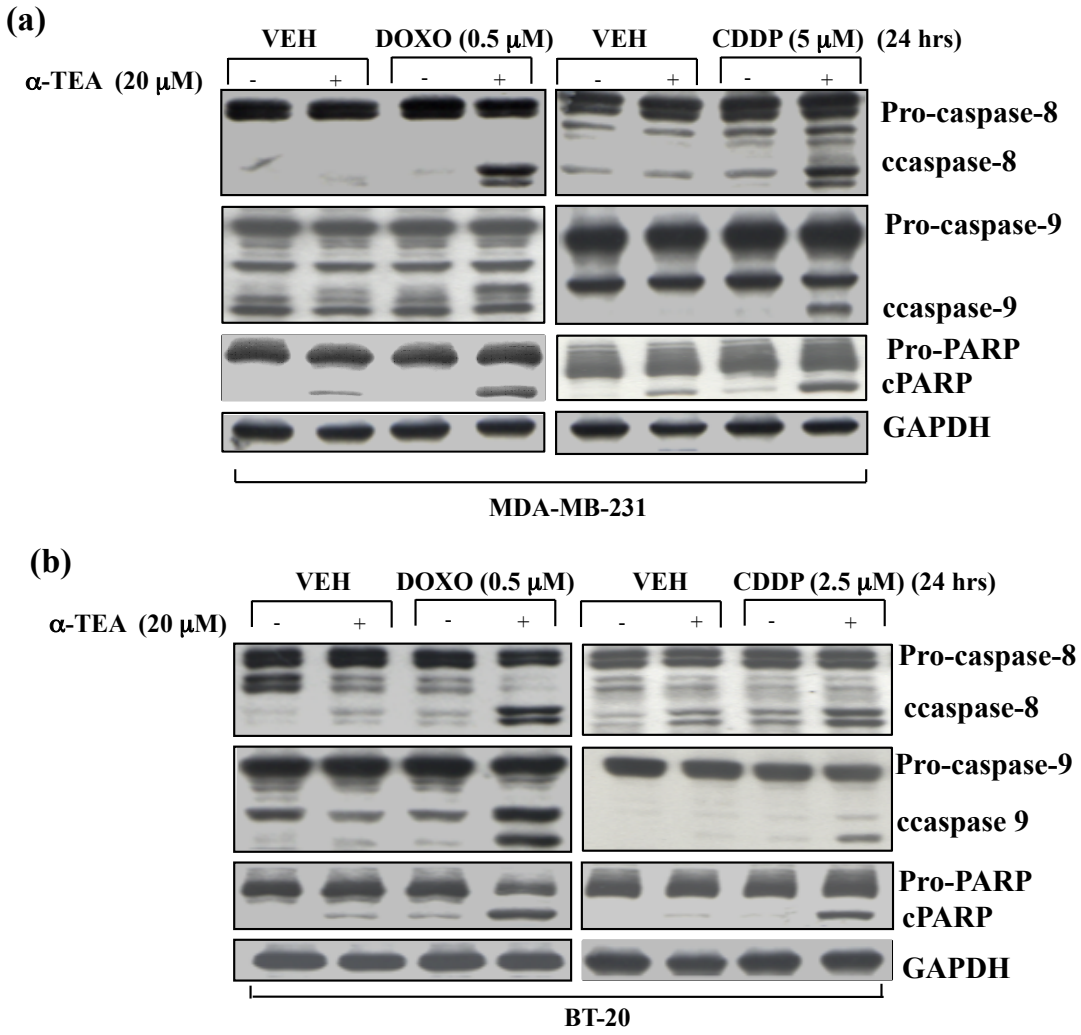


Figure 25. α-TEA cooperates with DOXO and CDDP to induce cleavage of caspase-8, -9 and PARP. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Western blot analyses were used to detect PARP, caspase-8, and caspase-9 cleavage (Fig 25a & b). Data are representative of at least three independent experiments.

p73 protein level is up-regulated by combinations of α-TEA + DOXO or CDDP and involved in combination induced apoptosis.

Since DOXO and CDDP as well as α-TEA have been shown to induce p73 up-regulation in breast cancer cells [Gong et al., 1999, Wang et al., 2008], the combination of α-TEA plus DOXO or CDDP was investigated for ability to cooperatively enhance p73 protein expression. Single treatments with DOXO, CDDP or α-TEA at sub-apoptotic levels for 24 hrs slightly increased p73 protein expression above control levels; whereas, combinations at the same levels markedly enhanced p73 protein expression in comparison with single treatments in both MDA-MB-231 and BT-20 cells (Figure 26 A and B). siRNA to p73 significantly reduced the ability of combination treatments to induce apoptosis as determined by Annexin V (Figure 26 C) and PARP analyses (Figure 26 D) in MDA-MB-231 cells. Western blot data show that siRNA to p73 effectively silenced p73 protein expression (Figure 26 D). These data indicate that p73 activation by combination treatments is critical for induction of cell death by apoptosis.

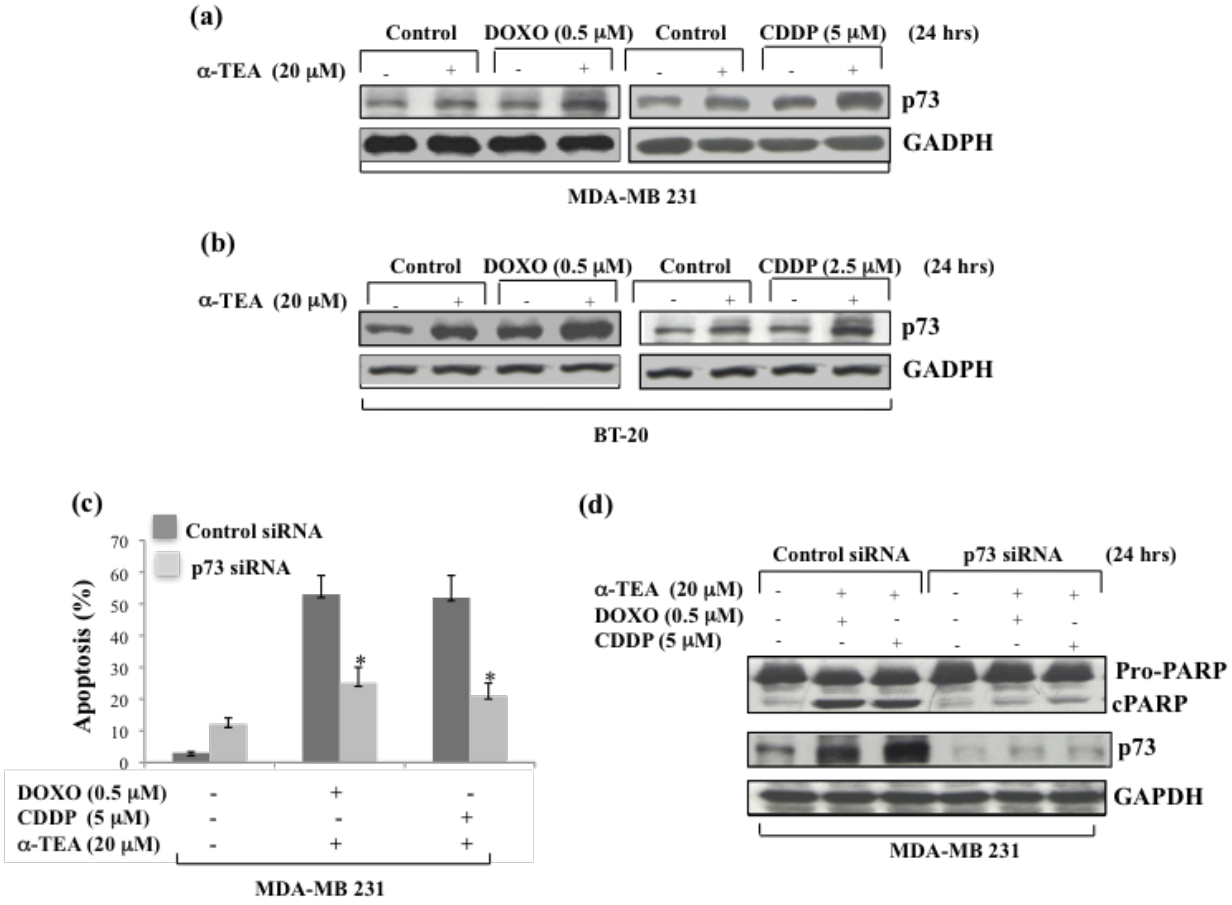


Figure 26. p73 is up-regulated by combination treatments and involved in combination induced apoptosis. MDA-MB-231 and BT-20 cells were treated with α-TEA, DOXO and CDDP alone or in combinations for 24 hrs. Western blot analyses were performed to detect protein levels of p73 with GAPDH serving as loading control (a & b). MDA-MB-231 cells were transfected with p73 siRNA or control siRNA for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. FACS/Annexin V assays were used to determine the percentage of apoptotic cells (c). Western blot analyses were used to verify the knockdown efficiency of p73 siRNA and the effect of p73 siRNA on combination induced PARP cleavage (d). Data in a, b and d are representative of at least two independent experiments and the data in c are expressed as mean ± SD from three independent experiments. *p<0.05=significantly different from control siRNA determined by *t*-test.

Combinations of α-TEA plus DOXO or CDDP up-regulate pro-apoptotic DR5, Fas, Bax, and Noxa mRNAs and proteins and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are mediated at least in part by p73

Published data show that p73 can regulate p53 dependent genes in p53 deficient cells [Vayssade et al., 2005]. To better understand the cellular events involved in p73 mediated apoptosis in combination treatments, mRNA and protein expression of p53 mediated pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and anti-apoptotic mediator Bcl-2 were examined. Combinations of α-TEA + DOXO or CDDP enhanced DR5, Fas, Bax and Noxa mRNA (Figure 4a & b) and protein expression (Figure 4c & d) and decreased Bcl-2 mRNA (Figure 27 A and B) and protein expression in MDA-MB-231 and BT-20 cells (Figure 27 C and D). siRNA knockdown of p73 was performed to determine if expression levels of these mediators were regulated by p73. siRNA to p73 in MDA-MB-231 cells effectively silenced p73 protein expression and blocked the ability of combinations to induce increased levels of DR5, Fas, Bax and Noxa protein, as well as to decrease Bcl-2 protein levels (Figure 27E). These data suggest that combination treatments induce up-regulation of pro-apoptotic mediators and down-regulation of anti-apoptotic mediator in a p73 dependent manner in p53 mutant, TNBC MDA-MB-231 and BT-20 cells. Recent studies in our lab show that DR5 pro-apoptotic signaling contributes to α-TEA-induced apoptosis

[Tiwary et al., 2010, Yu et al., 2010]. To determine if DR5 contributes to combination treatment induced apoptosis, DR5 was functionally knocked-down with siRNA. Data indicate that silencing DR5 protein expression blocks combination induced apoptosis as determined by PARP cleavage (Fig 27 F).

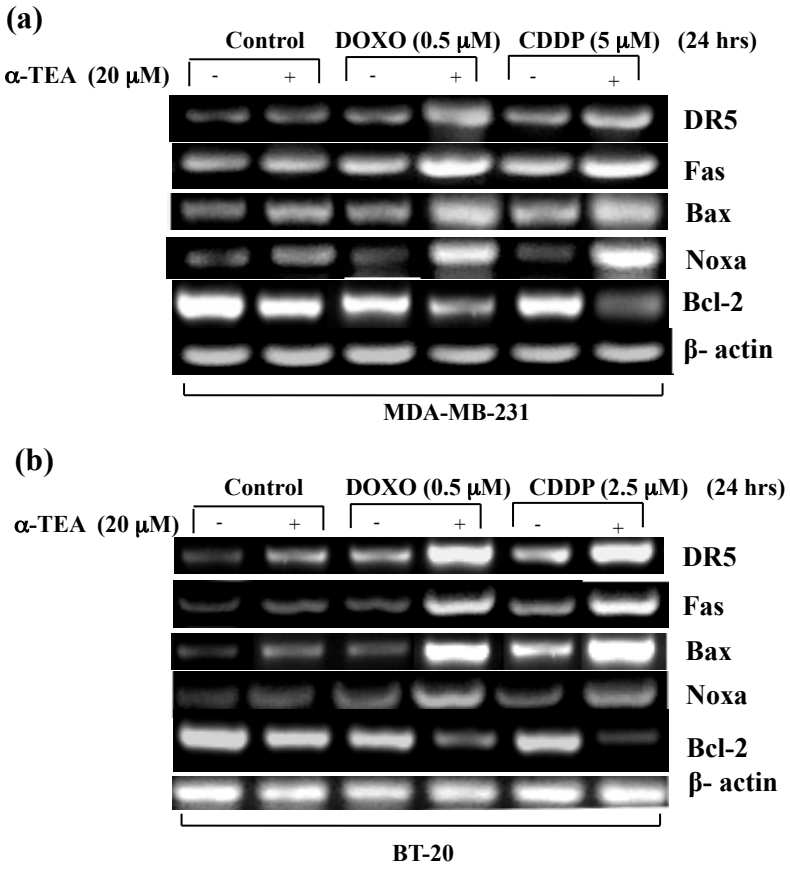


Figure 27. α-TEA cooperates with DOXO or CDDP to up-regulate mRNA and protein of pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are downstream targets of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. mRNA levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by RT-PCR with β-actin serving as loading control (a & b).

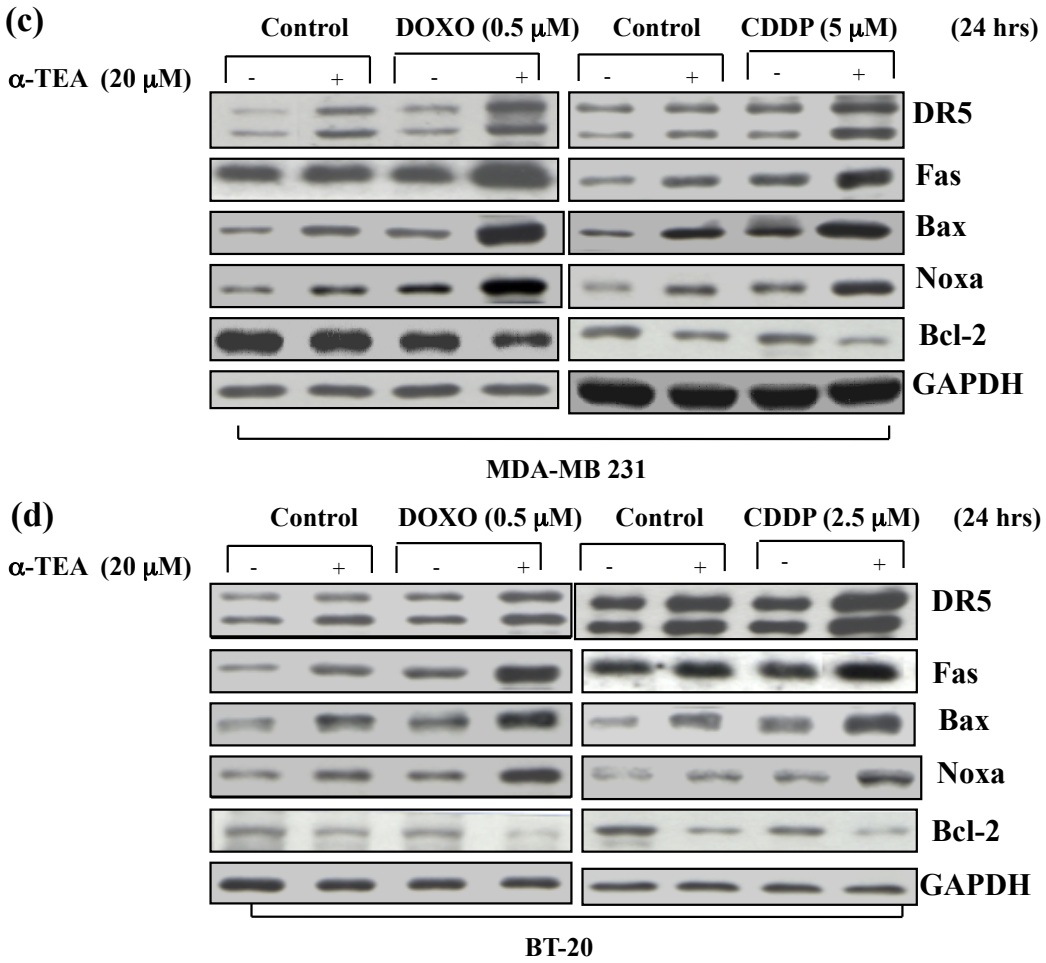


Figure 27. α-TEA cooperates with DOXO or CDDP to up-regulate mRNA and protein of pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are downstream targets of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Protein levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by western blot analyses with GAPDH serving as loading control (c & d).

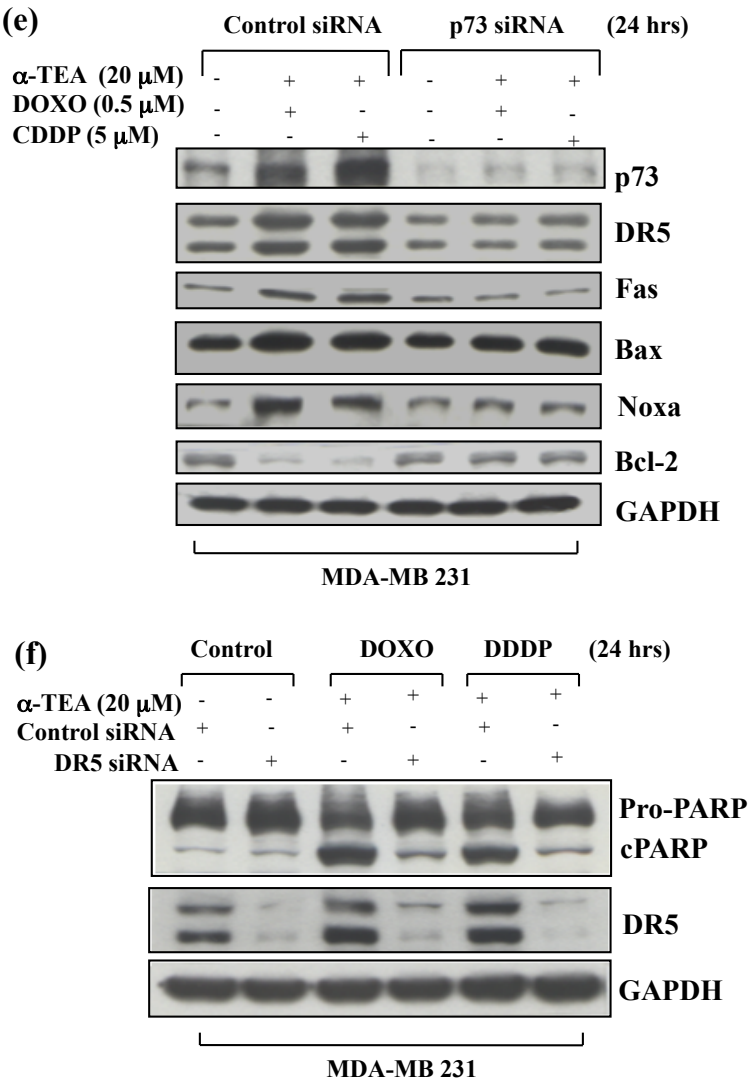


Figure 27. α-TEA cooperates with DOXO or CDDP to up-regulate mRNA and protein of pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are downstream targets of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. MDA-MB-231 cells were transfected with DR5 or control siRNA for 2 days and treated with combinations for 24 hrs. The same treated samples as Figure 26d were used to detect the effect of siRNA to p73 on combination induced increase in protein levels of DR5, Fas, Bax, Noxa and decrease in Bcl-2 by western blot analyses with GAPDH as loading control (e). Western blot analyses were used to determine the effect of siRNA to DR5 on combination induced PARP cleavage and to verify the knockdown efficiency of DR5 (f). Data are representative of at least two independent experiments.

α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which are upstream mediators of p73

Studies show that p73 can be up-regulated upon DNA damage via activation of c-Abl and JNK [Jones et al., 2007]. To understand how p73 is activated by the combination treatments, phosphorylated levels of c-Abl and JNK2/1 were examined. Combinations of α-TEA plus DOXO or CDDP induced increased levels of pc-Abl (Tyr-245) and pJNK2/1 in both cell lines (Figure 28 A and B). siRNA knockdown of c-Abl or JNK significantly reduced the ability of combination treatments to induce apoptosis in MDA-MB-231 cells as determined by Annexin V (Figure 28 C) and PARP cleavage (Figure 28 D). siRNA treatments blocked the ability of combination treatments to increase protein levels of p73 and block the ability of combination treatments to increase protein levels of DR5, Fas, Bax and Noxa, and to decrease level of Bcl-2 (Figure 28 D). siRNA to c-Abl blocked the ability of combination treatments to induce increased levels of pJNK; whereas, siRNA to JNK had no effect on the ability of combination treatments to induce increased levels of pc-Abl (Tyr 245) (Figure 28 D). These data show that activation of p73 is mediated by c-Abl and JNK in the combination treatments, and suggest that c-Abl, in part, regulates the phosphorylation status of JNK.

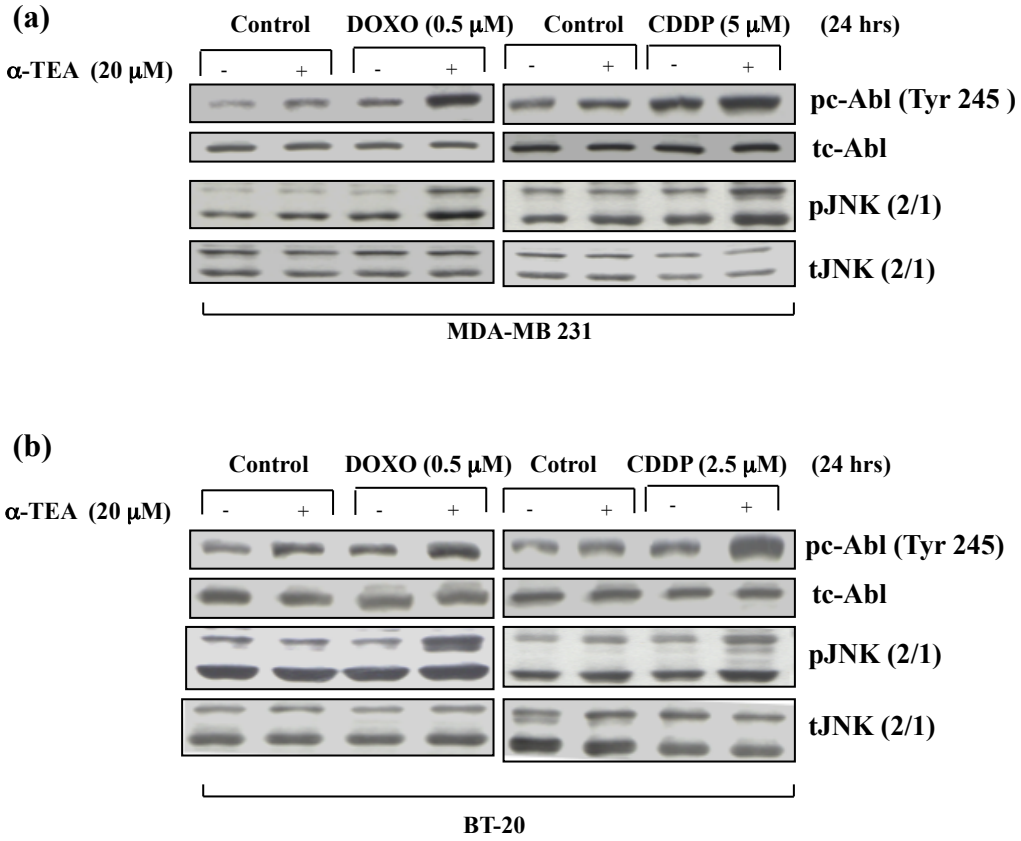


Figure 28. α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which can serve as upstream mediators of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Protein levels of pc-Abl (Tyr-245), total c-Abl (tc-Abl), pJNK2/1, and total JNK2/1 (tJNK2/1) were determined by western blot (a & b). MDA-MB-231 cells were transfected with c-Abl and JNK siRNAs, as well as control siRNA for 2 days and treated with combination of α-TEA + DOXO or CDDP for 24 hrs. Apoptosis was determined by Annexin V/FACS (c). Western blot analyses were used to verify the knockdown efficiency of c-Abl and JNK siRNAs and the effect of c-Abl and JNK siRNAs on combination induced PARP cleavage, as well as p73 and p73 mediated DR5, Fas, Bax, Noxa and Bcl-2 (d). Data in c are expressed as mean $\pm$ SD from three independent experiments and the data in a, b, and d are representative of at least two independent experiments. *p<0.05=significantly different from control siRNA determined by t-test.

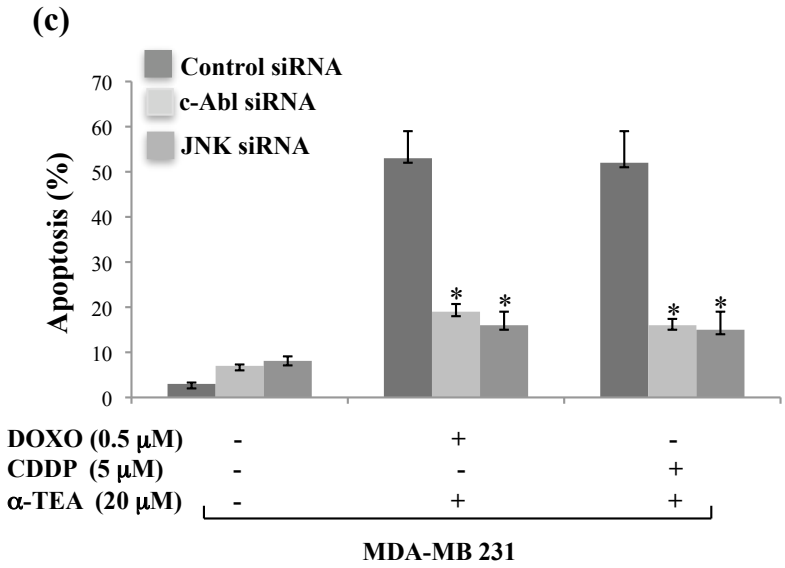


Figure 28. α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which can serve as upstream mediators of p73.

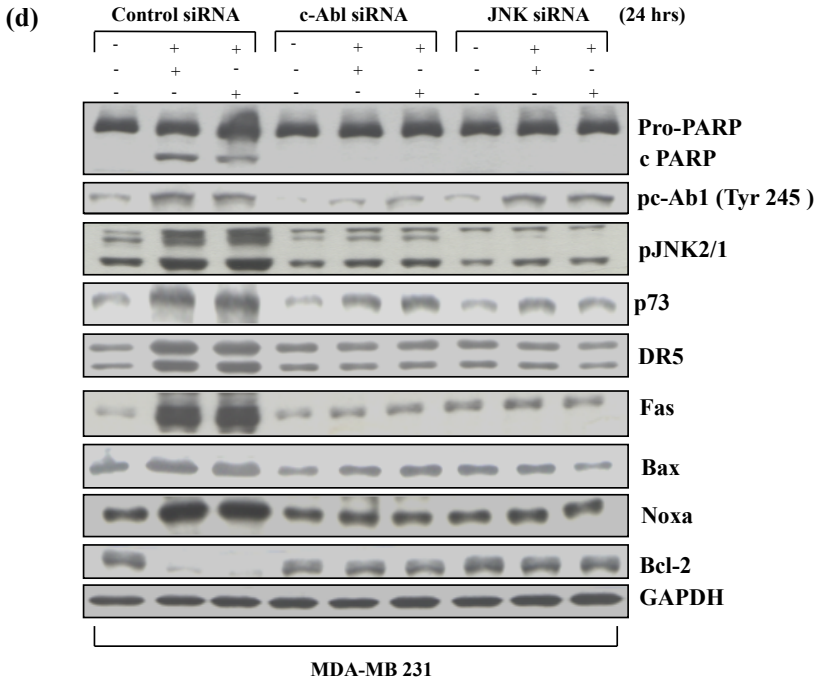


Figure 28. α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which can serve as upstream mediators of p73. MDA-MB-231 cells were transfected with c-Abl and JNK siRNAs, as well as control siRNA for 2 days and treated with combination of α-TEA + DOXO or CDDP for 24 hrs. Apoptosis was determined by Annexin V/FACS (c). Western blot analyses were used to verify the knockdown efficiency of c-Abl and JNK siRNAs and the effect of c-Abl and JNK siRNAs on combination induced PARP cleavage, as well as p73 and p73 mediated DR5, Fas, Bax, Noxa and Bcl-2 (d). Data in c are expressed as mean $\pm$ SD from three independent experiments.

Yap is involved in combination induced apoptosis

Since Yap, a transcriptional co-activator Yes-associated protein, can interact with p73, resulting in enhanced p73 transcriptional activity [Strano et al., 2001] and stability [Levy et al., 2007], we determined if Yap contributes to combination induced apoptosis and increased p73 expression. siRNA knockdown of Yap significantly reduced the ability of combination treatments to induce apoptosis as measured by annexin V analyses (Fig 29 A) and western blot analyses of PARP cleavage (Fig 29 B). siRNA to Yap effectively reduced Yap protein levels and blocked combination treatment effects on p73 protein expression, as well as combination effects on DR5, Fas, Bax, Noxa and Bcl-2 protein expression (Fig 29 B). These data show that Yap is a key player in combination treatment induced apoptosis mediated by p73.

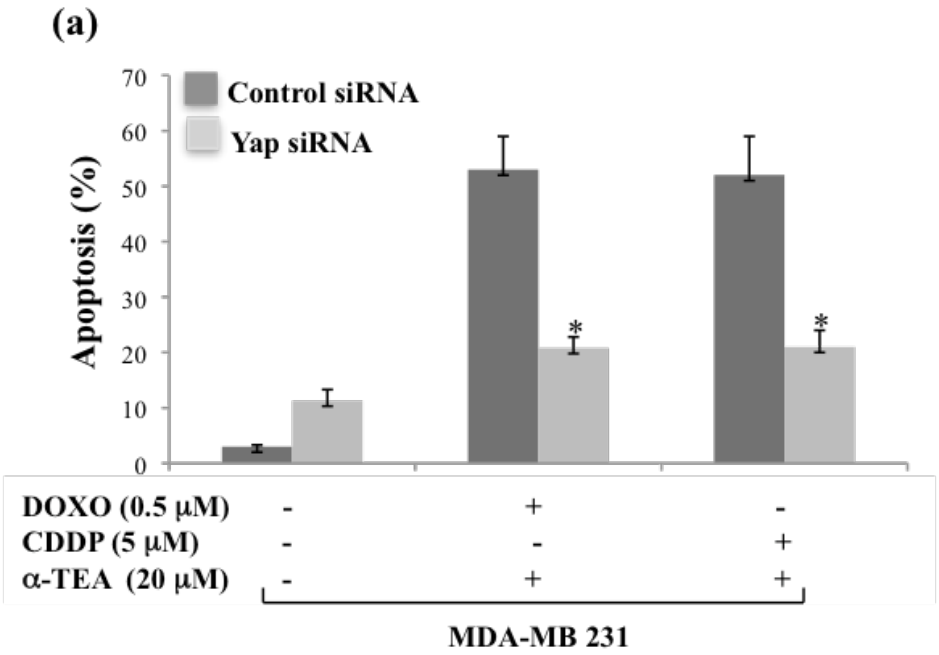


Figure 29. Yap is involved in combination treatment induced apoptosis.

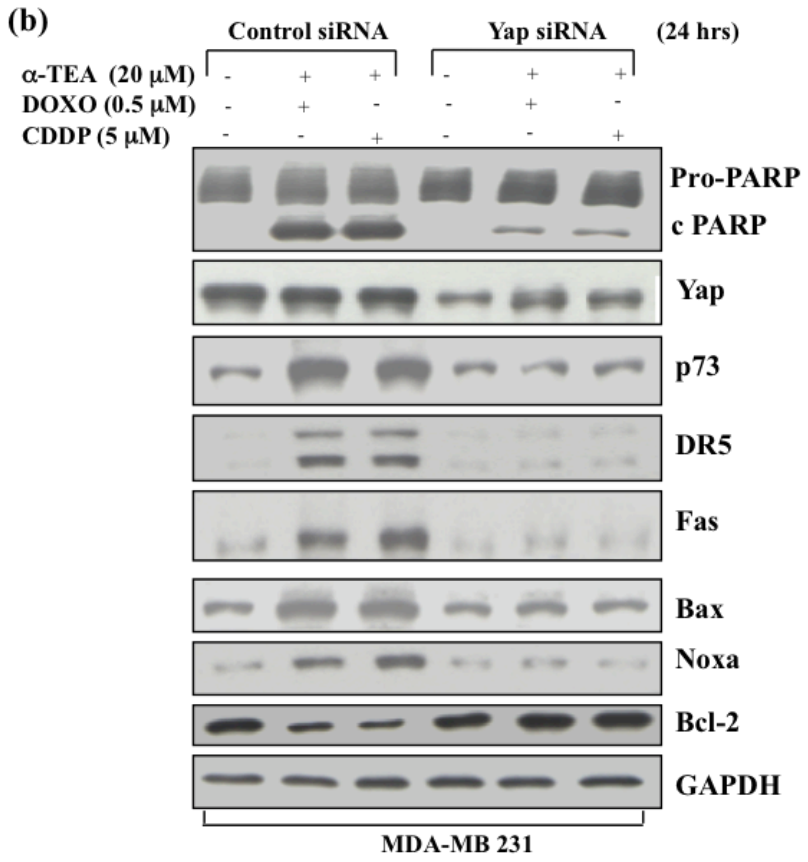


Figure 29. Yap is involved in combination treatment induced apoptosis. MDA-MB-231 cells were transfected with Yap siRNA or control siRNA for 2 days and treated with combinations for 24 hrs. Apoptosis was determined by Annexin V/FACS (a). Western blot analyses were used to verify the knock down efficiency of Yap siRNA and the effect of Yap siRNA on combination induced PARP cleavage, as well as p73 and p73 mediated DR5, Fas, Bax, Noxa and Bcl-2 (b). Data in b are representative of at least two independent experiments. Data in a are expressed as mean $\pm$ SD from three independent experiments. *$p<0.05$=significantly different from control siRNA determined by *t*-test.

Combination treatments induce Yap nuclear translocation, which is associated with suppression of phosphorylation of Akt and Yap.

Yap activity can be regulated by c-Abl via phosphorylation of Yap at Tyr-357, leading to its stabilization and higher affinity for p73 [Downward et al., 2008]. Furthermore, Yap can be negatively regulated by Akt [Basu et al., 2003]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization via promoting Yap binding with 14-3-3 resulting in inactivation of Yap [Basu et al., 2003]. Since α-TEA has been shown to decrease pAkt in prostate [Jia et al., 2008], ovarian [Shun et al., 2010], and breast cancer cells [data not shown] we examined the effect of combination treatments on Yap nuclear translocation, as well as pAkt and pYap expression. Combination treatments of MDA-MB-231 cells induced increased levels of Yap protein in the nuclear fraction and reduced levels of Yap protein in the cytoplasmic fraction. Histone 1 and GAPDH were used to evaluate purity of nuclear and cytoplasmic fractions, respectively, and served as lane load controls (Fig 30 A). Furthermore, data show that DOXO and CDDP increased pAkt and pYap protein expression while α-TEA cooperated with DOXO or CDDP to suppress pAkt and pYap in MDA-MB-231 (Fig 30 B). These data suggest that Yap nuclear translocation may partially contribute to p73 mediated effects and that combination treatment down-regulation of pAkt correlates with decreased levels of pYap. To assess the role of Akt in DOXO and

CDDP induced p73 protein expression, we examined the impact of PI3K/Akt inhibitor (wortmannin) on DOXO and CDDP induced p73 protein expression. Data show that wortmannin enhanced DOXO and CDDP induced up-regulation of p73 protein expression (Fig 30 C), indicating a role for Akt in DOXO and CDDP increase in p73 expression. Data also show that wortmannin blocked DOXO and CDDP induced up-regulation of pAkt and pYap (Fig 30 C), suggesting that suppression of pAkt enhances DOXO and CDDP induced p73 expression via down-regulation of pYap.

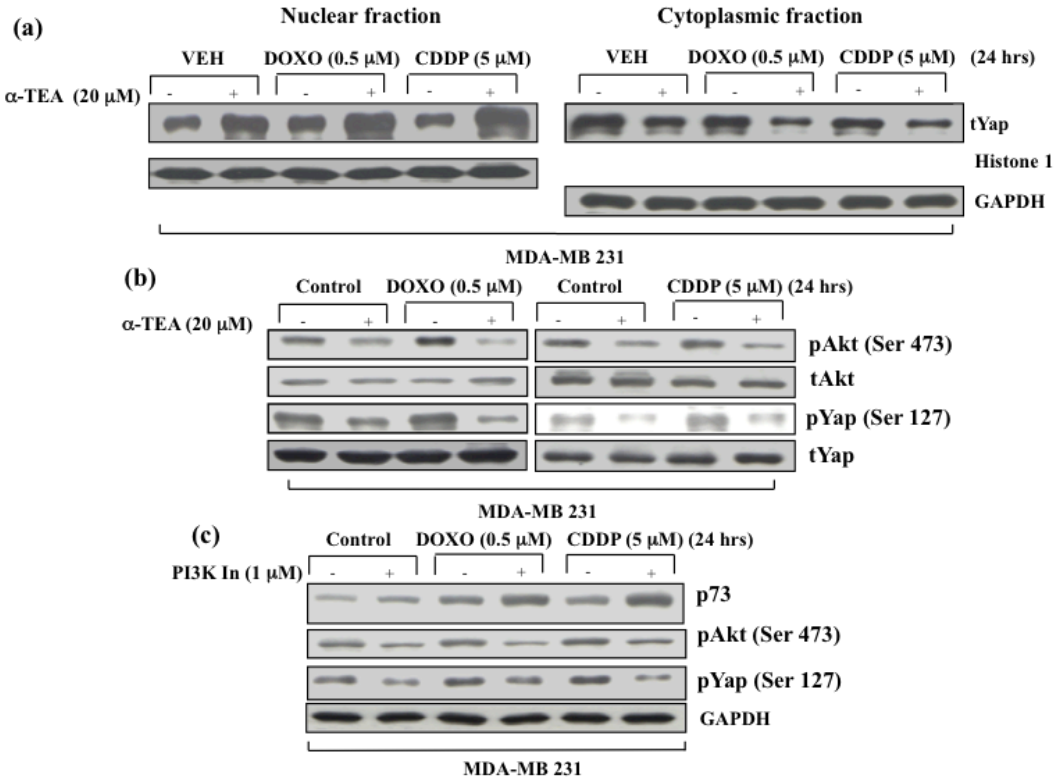


Figure 30. Combination treatments induce Yap nuclear translocation, which is associated with suppression of phosphorylation of Akt and Yap. Isolated cytosolic and nuclear fractions from MDA-MB-231 cells treated with α-TEA plus DOXO or CDDP were used to detect Yap tanslocation from cytosol to the nucleus by western blot analyses (a). MDA-MB-231 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Protein levels of pAkt and pYap were determined by western blot analyses with total Akt and Yap serving as controls (b). MDA-MB-231 cells were pre-treated with Akt inhibitor wortmannin at 1 μM or DMSO for 2 hrs followed by treatments with DOXO or CDDP for 24 hrs. Protein levels of p73, pAkt and pYap were determined by western blot analyses (c). Data are representative of at least two independent experiments.

4.4. Discussion

p73 is an important target for treating p53 mutant cancers [Chung et al., 2010; Irwin et al., 2004]. The novel findings in this study are: (i) α-TEA, a potent anticancer analog of vitamin E, synergizes with DNA damaging agents doxorubicin and cisplatin to induce apoptosis of human p53 mutant, triple negative human breast cancer MDA-MB-231 and BT-20 cells via targeting p73, (ii) combination treatments result in p73 dependent up-regulation of pro-apoptotic DR5, Fas, Bax and Noxa and down-regulation of anti-apoptotic mediator Bcl-2, all of which are p53 mediated apoptotic related genes, (iii) p73 and p73 mediated apoptotic events are regulated by c-Abl, JNK and Yap in combination treatments, and (iv) α-TEA downregulation of Akt partially contributes to p73 upregulation in combination treatments. Thus, our data, for the first time, identify α-TEA as a small bioactive anticancer agent that regulates p53 mediated genes via p53-independent mechanisms when combined with DNA damaging agents.

As a transcription factor, p73 shares structural and functional similarities with p53 [Irwin et al., 2004]. In cancer cells that express wild type p53, p73 has been reported to cooperate with p53 to induce apoptosis [Zhu et al., 2001]; whereas, in p53 mutant cancer cells p73 has been reported to induce apoptosis via activation of p53-inducible genes [Wang et al., 2006]. Typically, p53 induces apoptosis via

regulating apoptosis related genes; such as DR5, Fas, Bax, Noxa and Bcl-2 [Haupt et al., 2003; Bredow et al., 2007]. p73 is up-regulated in response to a subset of DNA-damaging agents, including DOXO, CDDP, camptothecin and etoposide [Moll et al., 2004]. Several p53 mediated apoptosis-related genes have been identified to be regulated by p73, such as Fas, Bax, Bim, Noxa and Puma [Amin et al., 2007; Melino et al., 2004]. However, whether DR5 is the downstream gene of p73 is not well documented. It has been reported that DR5 is regulated by p73 in H1299 human non small lung cancer cells [Zhu et al., 1998]. El-Deiry WS and co-workers reported that some isolated small molecules can activate p53 reporter activity, increase expression of p53 target genes such as p21(Waf1) and DR5 and induce apoptosis in p53-deficient colon cancer cells, in which some of them increased p53 reporter activity via p73 [Wang et al., 2006]. In addition, they characterized a derivative of the plant alkaloid ellipticine as an anticancer agent which induces p73 and DR5 protein expression in p53-deficient human colon carcinoma cell line [Lu et al., 2008]. However, both studies did not show direct evidence to confirm p73 regulation of DR5. To the best of our knowledge, no direct evidence showing that p73 regulates DR5 in human breast cancer cells has been published. In addition, there is no evidence to indicate that p73 regulates Bcl-2 in human breast cancer cells. Thus, this study, for the first time, demonstrates that both DR5 and Bcl-2 are mediated by p73 in p53 mutant, TNBC MDA-MB-231 and BT-20 human breast cancer cells treated with α-TEA

combination with DOXO or CDDP as determined by siRNA knockdown assays. Our previous data show that DR5 is involved in α-TEA induced apoptosis determined by siRNA knockdown study showing that siRNA to DR5 blocked α-TEA induced apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells [Tiwary et al., 2010; Yu et al., 2010]. Here, we demonstrated that DR5 is necessary, at least in part, for apoptosis induced by α-TEA combination treatments with DOXO or CDPP.

p73 has been reported to induce ER stress via transactivation of Scotin [Terrinoni et al., 2004]. Since DR5 and Bcl-2 expression can been regulated by ER stress via CHOP [McCullough et al., 2001; Yamaguchi et al., 2004] and α-TEA has been shown to induce ER stress and CHOP expression [Tiwary et al., 2010] we cannot rule out the possibility that p73 regulates DR5 and Bcl-2 via ER stress in combination treatments. Further studies are needed to address this issue.

p73 is predominantly regulated at the post-translational level in response to DNA damaging agents. c-Abl and JNK are activated by DNA damaging agents and both are involved in p73 activation [Jones et al., 2007]. DOXO and CDDP have been shown to regulate p73 via c-Ab1 [Gong et al., 1999]. c-Abl regulates p73 via different mechanisms; for examples, c-Abl can directly stabilize p73 via acetylation and phosphorylation of p73 [Costanzo et al., 2002] and c-Abl can stabilize p73 and enhance p73 transcriptional activity via phosphorylation of Yap [Levy et al., 2008]. JNK has been reported to stabilize p73 via phosphorylation of

p73 [Jones et al., 2007] and via JNK phosphorylation/activation of c-Jun [Toh et al., 2004]. In addition, JNK also activates p73 via enhancing c-Abl nuclear translocation [Yoshida et al., 2005]. In untreated cells, c-Abl is sequestered in the cytosol by 14-3-3 proteins. Upon exposure of cells to DNA damaging agents, JNK is activated and phosphorylates 14-3-3, resulting in the release of c-Abl into the nucleus, an event required for the induction of apoptosis in response to DNA-damaging agents [Yoshida et al., 2005]. Published and present data show that c-Abl also regulates JNK via phosphorylation, suggesting cross-talk between c-Abl and JNK [Kamath et al., 2007].

Yap (Yes-associated protein) is a transcriptional coactivator, which can interact with the p53 family member p73, resulting in an enhancement of p73's transcriptional activity [Strano et al., 2005] and stability [Levy et al., 2007]. A potential mechanism of the p73 protein stabilization was recently suggested by Levy et al. Namely, Yap competes with Itch, an E3 ubiquitin ligase involved in degradation of p73, for binding to p73 at the PPXY motif. Furthermore, Yap activity can be regulated by c-Abl via phosphorylation at Tyr-357, leading to more stable form of Yap that exhibits a higher affinity to p73 [Levy et al., 2007]. Yap can be negatively regulated by Akt [Lapi et al., 2008]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization since phosphorylation of Yap at Ser-127 promotes Yap binding with 14-3-3 [Basu et al., 2003]. Thus, Yap activation can be regulated in a positive manner by c-Abl

and in a negative manner by Akt. DNA damage can activate survival mediator Akt, resulting in reducing the anticancer efficacy of DNA damaging drugs. DOXO or CDDP induce activation of Akt in some cell lines [Belyanskaya et al., 2005]. Likewise, our data show that DOXO and CDDP induce elevated levels of pAkt not only in MDA-MB-231 (Fig 30), but also in MCF-7, MDA-MB-453 and BT-20 cells (Data not shown). As expected, Akt inhibitors have been reported to enhance the anticancer effect of DOXO in MDA-MB-231 cells [Wang et al., 2009]. Data reported here show that Akt inhibitor wortmannin enhanced DOXO and CDDP mediated increases in p73 protein expression, which is associated with down-regulation of pAkt and pYap in MDA-MB-231 cells. Taken together, these data suggest that Akt activation upon DNA damage may counteract p73 activation induced by JNK and c-Abl via inhibition of Yap nuclear translocation. Thus, our data suggest that Yap nuclear translocation plays an important role in p73 activation and that suppression of pAkt and its inhibitory phosphorylation of pYap contributes to enhanced Yap nuclear translocation in combination treatments.

How α-TEA induces p73 protein expression is not fully understood. Previously, we reported that JNK is involved in regulation of p73 in α-TEA treatment [Wang et al., 2008]. In this study, we found that α-TEA also induces increased levels of pc-Abl and Yap nuclear translocation, as well as suppress pAkt and pYap, suggesting that c-Abl and Yap, as well as downregulation of pAkt/pYap are also

involved in α-TEA induced apoptosis. Noxa has been identified as a downstream mediator of p73 in α-TEA induced apoptosis [Wang et al., 2008]. However, whether other p53 mediated genes, such as Fas, DR5, Bax and Bcl-2 are regulated by 73 following α-TEA treatment has not been investigated. Since recent data show that ER stress mediated CHOP contributes to α-TEA induced up-regulation of DR5 and down-regulation of Bcl-2 [Tiwary et al., 2010] it will be important for further studies to address whether both CHOP and p73 contribute to DR5 upregulation and Bcl-2 downregulation in α-TEA induced apoptosis.

Mechanisms mediating the combined anticancer effects of α-TEA + DOXO or CDDP are diverse and not completely understood. These studies identified p73 as a key player in combination treatment induced apoptosis. In addition, data show that c-Abl, JNK and Yap play roles in combination treatment induced activation of p73. It is important to note that although both α-TEA and DNA damaging drugs DOXO or CDDP induce increased levels of pc-Ab1 and pJNK, only α-TEA and the combination of α-TEA plus DOXO or CDDP induces Yap nuclear translocation which is associated with inhibition of pAkt (Ser-473) and Akt phosphorylated pYap (Ser-127). Furthermore, a PI3K/Akt inhibitor was shown to enhance DOXO and CDDP up-regulation of p73, which was also associated with down-regulation of pAkt and pYap. Taken together, these data suggest that down-regulation of pAkt and pAkt mediated inactive form of Yap play important roles in p73 activation and apoptosis in combination treatments. Thus, α-TEA

cooperates with DOXO or CDDP to induce p73 protein expression and apoptosis via not only activation of c-Abl and JNK, but also Yap, which may be regulated positively by c-Abl and negatively by Akt. Based on published reports and data presented here we proposed signaling events necessary for combination treatment induced apoptosis in p53 mutant, TNBC cells (Fig 31).

In summary, data demonstrated that α-TEA, a small bioactive lipid, cooperates with DNA damaging agents DOXO and CPPD to induce apoptosis in human breast cancer cells via targeting p53 inducible apoptotic related genes in a p73-dependent manner. These studies highlight the potential for activation of p73 as a promising target for treatment of p53 mutant, TNBC and identify α-TEA as an important candidate agent.

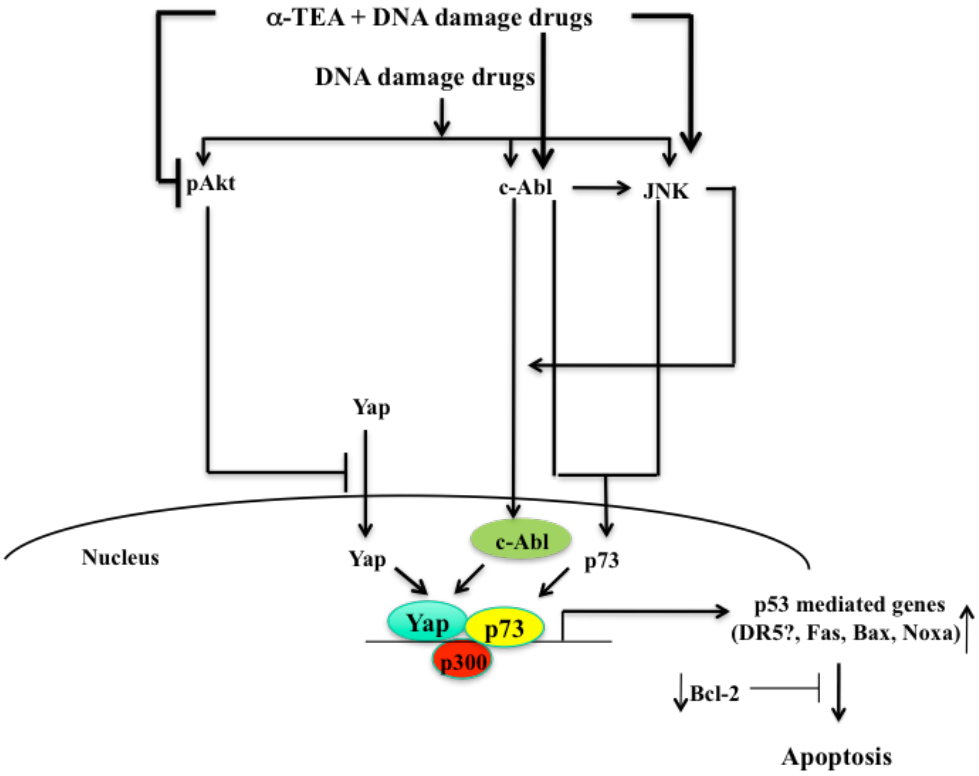


Figure 31. Proposed signaling pathways whereby the combination of α-TEA + DOXO or CDDP induces apoptosis in p53 mutant, triple negative MDA-MB-231 and BT-20 human breast cancer cells. p73 can be activated via multiple mechanisms and pathways including; (i) c-Abl and JNK can directly phosphorylate p73 to stabilize it, (ii) Yap in nucleus can bind with p73 to enhance its transcriptional activity and stability, and (iii) p73 can be transcriptionally regulated (not studied here). Yap can be regulated in a positive manner by c-Abl via phosphorylation enhancing its stability and transcriptional activity and in a negative manner by Akt via inhibiting translocation of Yap into the nucleus. c-Abl can directly phosphorylate JNK and JNK can enhance c-Abl nuclear translocation. Therefore, c-Abl, JNK and Yap play positive roles and Akt plays a negative role in p73 activation. Our data show that DNA damaging drugs DOXO and CDDP activated c-Abl and JNK, but also activated Akt, which can counteract c-Abl and JNK effects on activation of p73. Combination treatments not only act cooperatively to activate c-Abl and JNK, but also act cooperatively to inhibit pAkt and pYap (ser-127), leading to Yap nuclear translocation and p73 activation. In summary, combinations of α-TEA + DOXO or CDDP act cooperatively to up-regulate c-Abl/JNK, induce Yap nuclear translocation and down-regulate pAkt/pYap, leading to activation of p73 and up-regulation of p73 mediated pro-apoptotic factors mediators, and down-regulation of Bcl-2, thereby restoring DOXO and CDDP chemotherapeutic potential in p53 mutant, triple-negative breast cancers.

Chapter 5. α-TEA plus tamoxifen combination circumvent tamoxifen resistance via disruption of cholesterol-rich microdomains, suppression of pro-survival mediators and activation of endoplasmic reticulum stress

5.1.Introduction

Of the estimated 192,370 new cases of breast cancer diagnosed among women in the U.S. in 2009, approximately 70% of them will be breast tumors that are estrogen receptor alpha-positive (ER+) [Susan G Komen, Breast Cancer facts 2009]. Unfortunately, 40-50% of all ER+ breast cancer patients will either not respond to endocrine therapy (ET) (i.e. exhibit de novo resistance) or suffer cancer recurrence due to acquired ET resistance [Fabian, 2007]. Clearly, more basic information about how ET resistance can be circumvented is needed.

Tamoxifen (TAM) is a selective estrogen-receptor modulator (SERM) with estrogenic actions in endometrial tissue, adipose tissue and bone, and anti-estrogenic actions in breast tissue [Lewis et al. 2005]. Tamoxifen binds to and antagonizes ERα and has been the mainstay of ET in both early and advanced ER+ breast cancer patients for almost three decades. However, TAM resistance remains the major barrier for successful application in the clinic. Of the many possibilities, aberrant over-expression of growth factor signaling pathways is implicated as important contributors to both acquired and de novo TAM resistance [Musgrove et al., 2009; Hurvitz et al., 2008]. In TAM resistant breast

cancer cells, over-expression of receptor tyrosine kinases (RTK's) such as Her-1 and Her-2 crosstalk with membrane associated ER (mER), leading to ER independent cell proliferation, in which TAM acts as agonist [Arpino et al., 2008].

RRR-α-tocopherol ether-linked acetic acid analog (α-TEA), a unique small active lipid, has been shown to possess non-toxic anticancer properties both *in vitro* and *in vivo* [Latimer et al., 2009; Shun et al., 2004; Jia et al., 2008; Wang et al., 2008; Lawson et al., 2004; Anderson et al., 2004]. Molecular mechanistic studies show that α-TEA possesses dual functions to target cancer cells, namely, (i) activation of pro-apoptotic pathways including Fas/Fas L, ER stress mediated JNK/CHOP/DR5, p73, leading to caspase-8 and mitochondria dependent apoptosis and (ii) suppression of pro-survival/anti-apoptotic factors such as Her-1, Her-2, Akt/mTOR, ERK, c-FLIP, Bcl-2 and Survivin [Jia et al., 2008; Tiwary et al., 2010; Wang et al., 2008; Shun et al., 2010].

Lipid raft microdomains are characterized as lateral assemblies of glycosphingolipids and cholesterol that form liquid-ordered membrane phases, insoluble in cold non-ionic detergents, light buoyant density, all these attributes contribute to their detergent resistant structures. Recent reports have suggested that these cholesterol-enriched microdomains are highly expressed in tumor cells [Li et al., 2006; Hoessli et al., 2000]. Cholesterol lipid raft microdomains provide the necessary platforms for growth factors, RTKs, and their downstream mediators, such as Akt and ERK (RTK/Akt/ERK complex), to contact, crosstalk,

and activate [Marquez et al., 2006; Patra, 2008]. Therefore, cholesterol lipid raft domains are described as “survival pool” for signaling pro-survival pathways and promoting cell growth, and they are promising targets for cancer prevention and therapy.

Data presented here show that α-TEA + TAM combination acts cooperatively to circumvent TAM resistance in both acquired and de novo TAM resistant cell lines via activation of pro-apoptotic and suppression of pro-survival mediators. α-TEA's one-two punch for circumvention of TAM resistance is by downregulation of RTK/Akt/ERK-mediated ER-dependent and -independent pro-survival signaling, and by activation of ER stress JNK/CHOP/DR5/caspase-8-mediated mitochondria dependent apoptosis. Furthermore, data show that cholesterol lipid rafts play an important role in TAMR pro-survival mediators and TAM resistance, and targeting cholesterol lipid raft is involved in TAM + α-TEA circumvention of TAM resistance. Taken together, our data demonstrate the potential of α-TEA + TAM as a novel combination for preventing as well as circumventing TAM resistance in ER-positive human breast cancer, and provides new knowledge about mechanisms of endocrine therapy resistance that can be circumvented.

5.2. Material and Methods:

Chemicals α-TEA (F.W.=488.8) was prepared in our lab as described previously [Lawson et al., 2004]. Tamoxifen was purchased from Calbiochem (La Jolla, CA). Methyl-β-cyclodextrin was purchased from Sigma.

Cell Culture

TAM-sensitive MCF-7/parent (MCF-7/TAMS) and acquired TAM resistant MCF-7/TAMR cells are kind gifts from Dr. Linda deGraffenried at UT Austin. TAM resistant MCF-7/Her2-18 cells are a kind gift from Dr. Mien-Chie Hung at MD Anderson in Houston. TAM sensitive were cultured as described previously [Wang et al., 2008]. MCF-7/TamR cells were grown in steroid-depleted phenol red free IMEM with 10% charcoal stripped serum supplemented with Tamoxifen (10^{-7}M). Three days prior to treatment, cells were grown in steroid-depleted phenol red free IMEM with 10% charcoal stripped serum supplemented with E_2 (10^{-9}M) for MCF-7/S and without E_2 for TAMR cells. During treatments, serum was reduced to 2% without TAM.

Western Blot Analyses

Whole cell protein extracts were prepared and western blot analyses were conducted as described previously [Jia et al., 2008]. Proteins at 20-50 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the

following proteins were used: poly (ADP-ribose) polymerase (PARP), c-FLIP, CHOP, GRP-78, pERK and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA), Bid (Pharmigen, Rockville, MD), Caspase-8, Caspase-9, DR5, phospho-IGF1R-β, IGF1R-β, phospho-Her1, Her-1, phospho-Her2, Her-2, FASN, phospho ER-α (ser-118), phospho ER-α (ser-167), phospho-Akt (ser-473), Akt and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA).

RNA Interference

A scrambled RNA duplex purchased from Ambion (Austin, TX) that does not target any known genes was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of MCF-7/TamR cells with siRNA to DR5, CHOP, JNK, Akt-1, c-FLIP or control (Ambion, Austin, TX) was performed in 100 mm cell culture dishes at a density of 2 x 10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After one day expose to transfection conditions, the cells were re-cultured in 100 mm dish at 2 x 10^6 cells/dish and incubated for one day followed by treatments.

Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assay (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Fluorescence was measured using a Fluorescence Activated Cell Sorter (FACS) 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.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey test was used for comparison of more than two treatments and a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p<0.05$.

5.3. Results

Constitutive active survival mediators in TAM resistant breast cancer cells that are enhanced by TAM treatment

Molecular profile of survival mediators in both TAMR cell lines, in comparison to TAMS parental cells, cultured with and without TAM, was determined by western blot analyses. Growth factor receptors Her-1, Her-2 and IGF1R (phosphorylated form as well as total protein), and their downstream mediators; pAkt, pERK1/2, pmTOR, c-FLIP, as well as pER-α (ser-167, ser-118) are constitutively expressed in TAMR cells in comparison with their parental TAMS cells (Fig 32). TAM treatment suppresses these growth factor mediators in TAMS cells and enhances their expression in TAMR cell lines (Fig 1). These data demonstrate that survival mediators are highly expressed in TAMR, in comparison to TAMS cells, and that TAM acts as an agonist to further stimulate these constitutively activated survival mediators, defining the molecular profile of rapidly dividing TAMR breast cancer cells with and without TAM treatment.

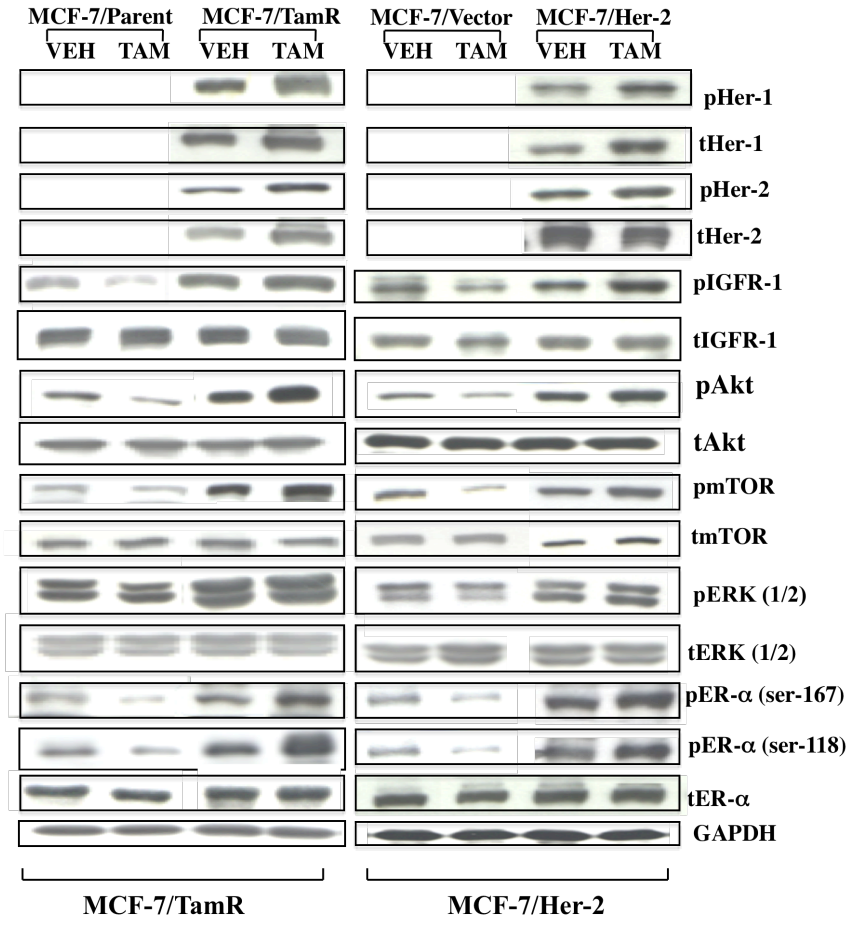
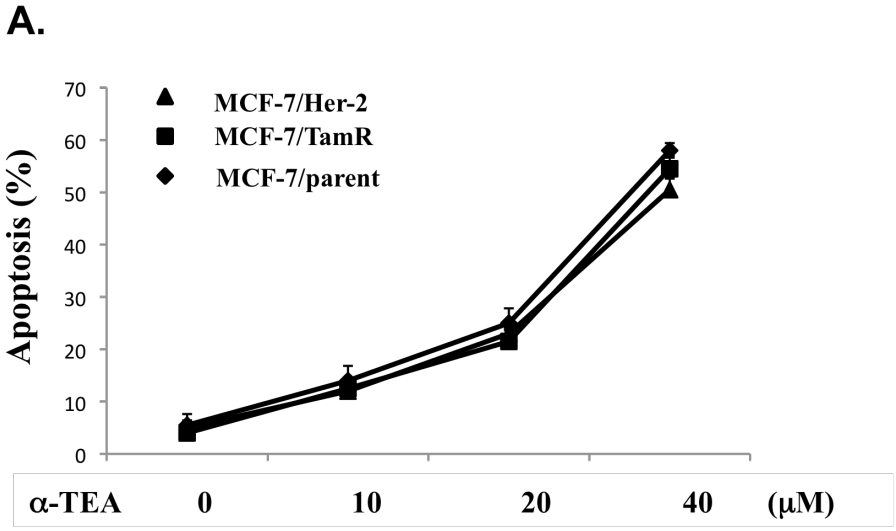
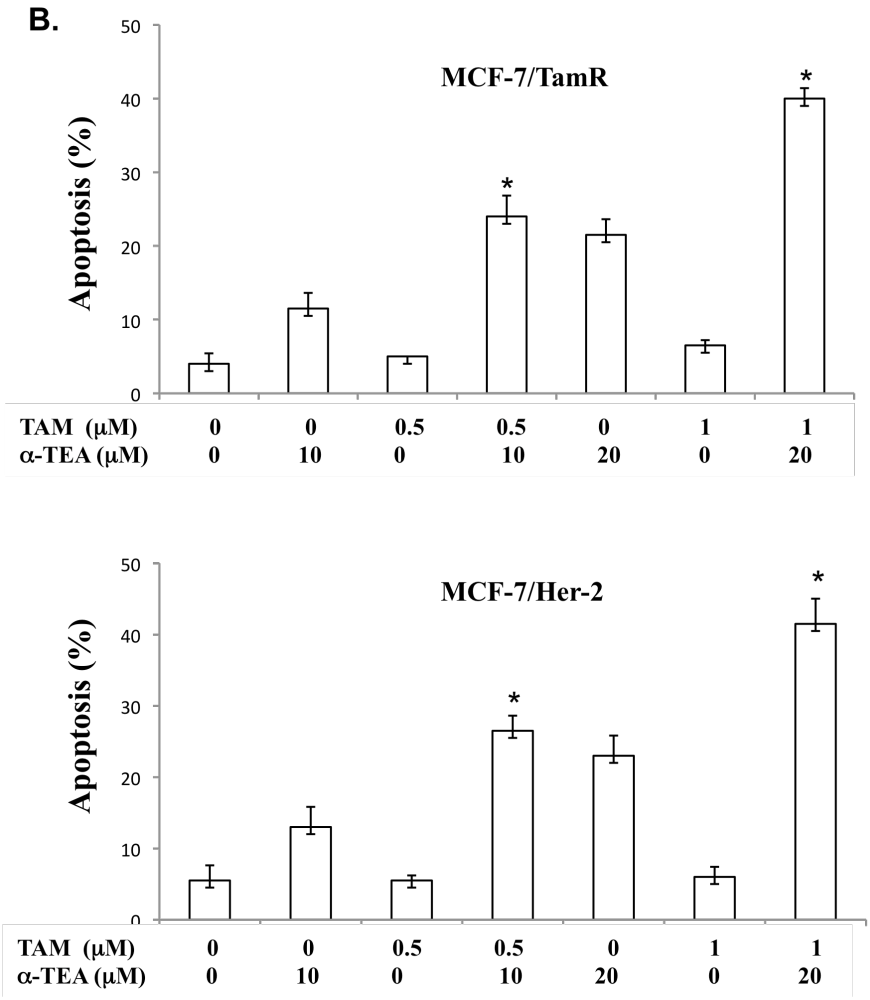


Figure 32: Untreated (vehicle) as well as TAM-treated TAM resistant MCF-7/TamR and MCF-7/Her-2 cells express elevated levels of pro-survival proteins (phosphorylated and total) in comparison with TAM sensitive MCF-7/parent isogenic and MCF-7/Vector cells respectively, either treated or untreated. In comparison to TAM treated sensitive cells, TAM resistant cells express higher levels of active form (phosphorylated) and total protein of pHer-1/total Her-1, pHer-2/total Her-2, pIGF1R, pAkt, pERK and pmTOR as well as pER-α. (Note: ER-a is phosphorylated at ser-167 and ser-118 by Akt and ERK, respectively), and total FASN protein. Western blot analyses were performed on whole cell lysates collected from MCF-7/parent and MCF-7/TamR and MCF-7/Vector and MCF-7/Her-2 cells cultured for three days in steroid-depleted media prior to treatment with TAM (1 μM) or vehicle (VEH) with E_2 (10^{-9}M) for TAM sensitive cells, for two days to compare signaling molecules in proposed pro-survival pathways. Phospho-protein and total protein levels were normalized by total protein and GAPDH, respectively and the relative densitometry values are expressed using MCF-7/parent vehicle control levels as 1. (Note: pHer-1/tHer-1 and pHer-2 were not detected in the MCF-7 TAM sensitive cells, therefore, the relative density in the TAM treated resistant cells was compared with TAM resistant vehicle control). Data are representative of three independent experiments.

α-TEA cooperates with TAM to induce apoptosis in TAM resistant cell lines

α-TEA significantly enhanced levels of apoptosis in a dose response manner in both TAMR cells and TAMS cells (Fig 33 A). TAM alone at 10 μM did not induce apoptosis in TAMR cells (data not shown). Combination of α-TEA + TAM significantly increased levels of apoptosis in MCF-7/TamR and MCF-7/Her-2 cells in comparison to vehicle control and single treatments (Fig 33 B), and PARP cleavage (Fig 33 C) with CI (combination index) value shown in Table 3, indicating synergistic actions of α-TEA + TAM in induction of apoptosis. The cooperative actions of the combination treatment are further confirmed by induction of increased levels of cleaved caspase-8 and -9 (Fig 33 C), showing that the combination of α-TEA + TAM induces caspase-8 and caspase-9 dependent apoptosis in both TAMR cell lines.




B.
MCF-7/TamR
Apoptosis (%)
50
40
30
20
10
0
*
*
TAM (μM) 0 0 0.5 0.5 0 1 1
α-TEA (μM) 0 10 0 10 20 0 20
MCF-7/Her-2
Apoptosis (%)
50
40
30
20
10
0
*
*
TAM (μM) 0 0 0.5 0.5 0 1 1
α-TEA (μM) 0 10 0 10 20 0 20

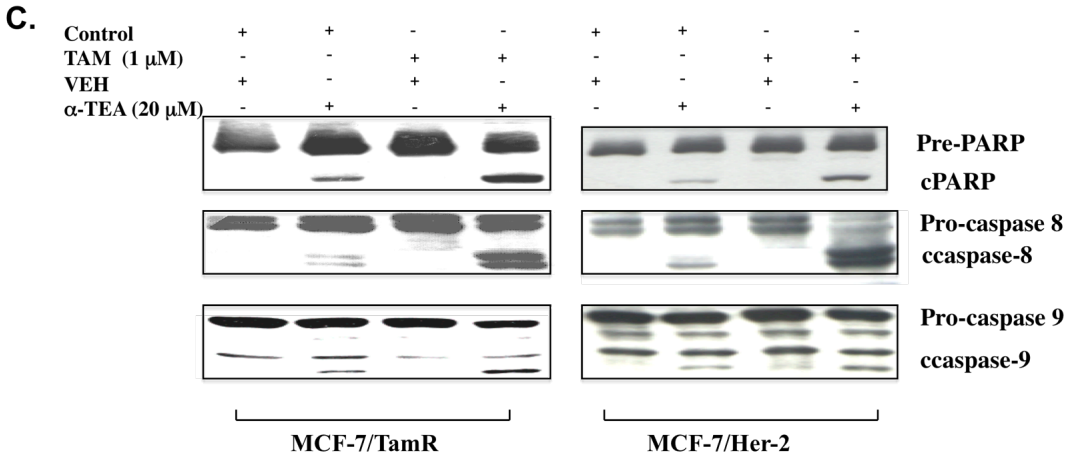


Figure 33: A. α-TEA induces apoptosis in MCF-7, MCF-7/TamR and MCF-7/Her-2 cells at 10, 20 and 40 μM at 1 day. **B.** α-TEA + TAM can circumvent TAM resistance in TAM resistant cells via induction of apoptosis. MCF-7/TamR and MCF-7/Her-2 cells were treated with α-TEA (20 μM), TAM (0.5 or 1 μM) and in combination as indicated for 1 day. Apoptosis was determined by FACS analyses after annexin V/PI staining. Data are depicted as mean ± SD of three individual experiments. *significant difference between α-TEA alone and control, **significantly different between α-TEA + TAM treated cells and α-TEA treated cells, and *** significant difference between α-TEA + TAM (0.5 μM) treated cells and α-TEA + TAM (1 μM) treated cells, $p<0.05$. **C.** MCF-7/TamR and MCF-7/Her-2 cells were treated with α-TEA (20 μM), TAM (1 μM) and in combination as indicated for one day for Western blot analyses. Data show that α-TEA, but not TAM, induces low level of cleaved PARP, caspase-8 and caspase-9 and the combination of α-TEA + TAM cooperatively activates cleavage of caspases-8 and -9. Data represent at least two separately conducted experiments.

Table 3 Combination Index (CI) of apoptosis [a]

	α-TEA:TAM Ratio [b]	CI [c]			Mean ± SD [d]	
		ED50	ED75	ED90		
MCF-7/TamR	20:1	0.5	0.48	0.46	0.48 ± 0.02	Synergism [e]
MCF-7/Her-2	20:1	0.33	0.26	0.20	0.26 ± 0.19	Synergism [e]

[a]MCF-7/TamR and MCF-7/Her-2 breast cancer cells were treated with different concentrations of α-TEA and TAM alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining /FACS assay as described in Material and Methods.
[b]The ratio for the concentrations that were used in combination treatments were determined from the data in Fig1.
[c]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).
[d]The mean ± SD is calculated from the CI values of ED50, ED75 and ED90.
[e]CI values < 1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

α-TEA + TAM act cooperatively to induce endoplasmic reticulum (ER) stress mediated apoptosis

Combination of α-TEA + TAM induced increased levels of ER stress marker, GRP78 and ER stress mediated pro-apoptotic factors, pJNK (2/1), CHOP and DR5 (L/S) in both TAMR cell lines (Fig 34 A). siRNAs to JNK, CHOP and DR5 block combination induced increases in pJNK (2/1), CHOP and DR5 (L/S), as well as apoptosis detected by PARP cleavage in the MCF-7/TamR cells (Fig 34 B). These data suggest that combination of α-TEA + TAM enhances α-TEA induced ER stress mediated apoptosis. TAM induces ER stress namely upregulation of GRP-78 and CHOP at high doses (10 μM and above) of MCF-7/TamR (data not shown).

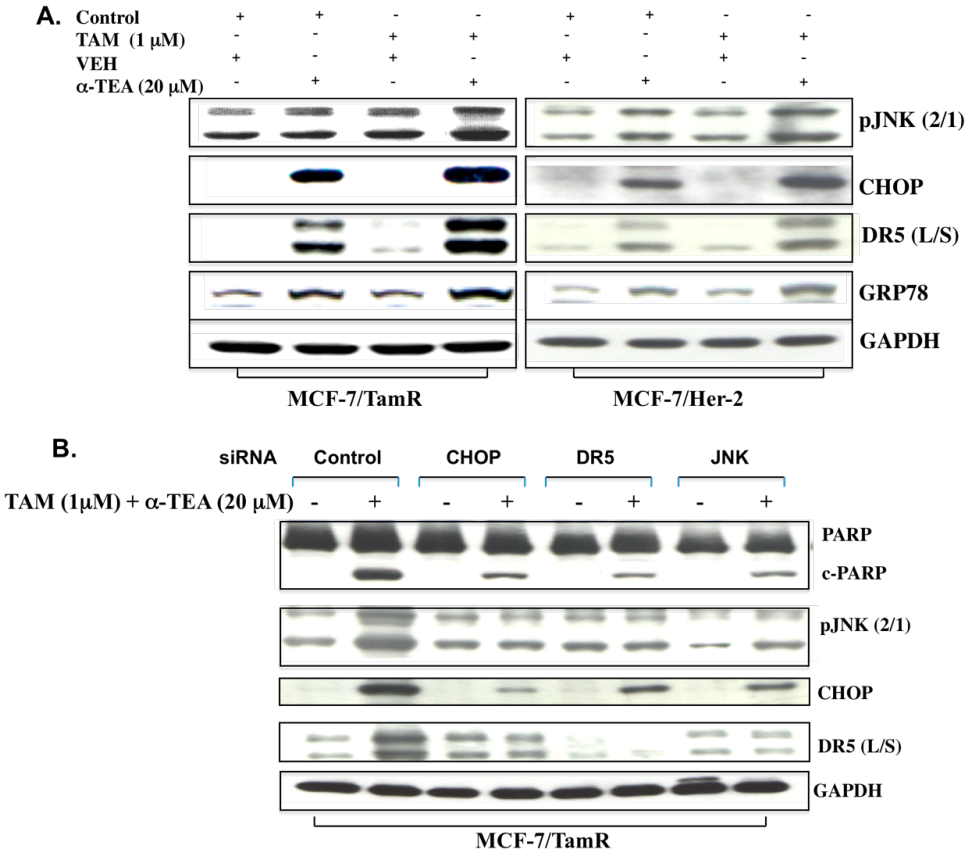
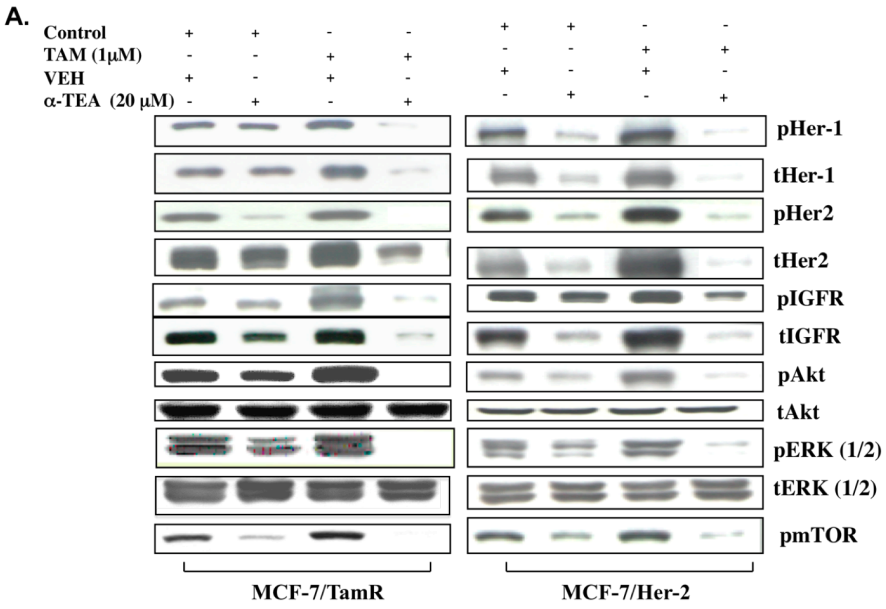


Figure 34: A. MCF-7/TamR and MCF-7/Her-2 cells were treated with a-TEA (20 μM), TAM (1 μM) and in combination as indicated for 1 day for Western blot analyses. α-TEA upregulate pJNK1/2, CHOP, DR5 and ER stress marker GRP78, while TAM upregulates lower levels of pJNK1/2, DR5 and ER stress marker GRP78 in comparison with a-TEA, and the combination of a-TEA + TAM cooperatively upregulates pJNK1/2, total protein levels of CHOP, DR5, as well as GRP78. **B.** MCF-7/TamR cells were transiently transfected with siRNA to CHOP, DR5, JNK as well as a nonspecific negative control siRNA (labeled Control) using our standard lab procedures. Transfected cells were cultured for three days prior to collection of whole cell lysates for Western immunoblot analyses. siRNA to CHOP, DR5 and JNK blocked a-TEA + TAM induced apoptosis as indicated by PARP cleavage. Proteins levels of CHOP, DR5 and JNK were checked to verify transfection efficiency. Data represent at least three separately conducted experiments.

TAM alone enhances pro-survival/anti-apoptotic factors whereas, α-TEA plus TAM circumvents TAM resistance by cooperatively suppressing pro-survival/anti-apoptotic factors

TAM alone increases the expression of pro-survival/anti-apoptotic growth factor receptors Her-1, Her-2 and IGFR in both total and phosphorylated protein levels, their downstream mediators pAkt, pERK (1/2) and pmTOR (Fig 35 A), and phosphorylated ER-α at ser-118 and ser-167, as well as anti-apoptotic factors c-FLIP and Bcl-2 in both cell lines (Fig 35 B). Importantly, α-TEA alone, and more extensively when in combination with TAM, blocks the expression of these pro-survival/anti-apoptotic mediators (Fig 35 A & B). These data demonstrate that TAM plays an agonist role in regulating growth factor/anti-apoptotic mediators when treated alone and plays an antagonistic role in regulating growth factor mediators when tested in combination with α-TEA, indicating that α-TEA reverses TAM resistance.


A.
Control
TAM (1μM)
VEH
α-TEA (20 μM)
pHer-1
tHer-1
pHer2
tHer2
pIGFR
tIGFR
pAkt
tAkt
pERK (1/2)
tERK (1/2)
pmTOR
MCF-7/TamR
MCF-7/Her-2

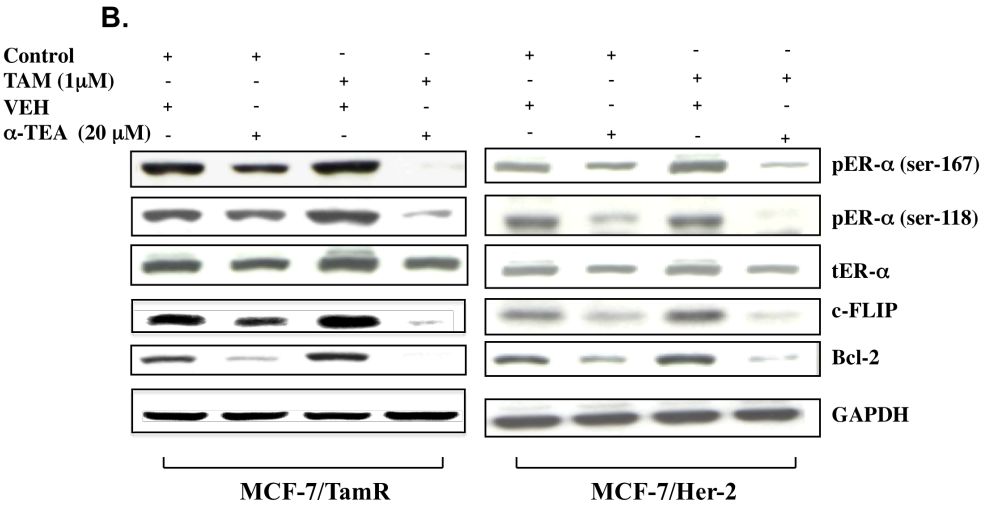

B.
Control
TAM (1μM)
VEH
α-TEA (20 μM)
pER-α (ser-167)
pER-α (ser-118)
tER-α
c-FLIP
Bcl-2
GAPDH
MCF-7/TamR
MCF-7/Her-2

Figure 35: Western immunoblot analyses using aliquots of cell lysates used in Figure 3A above were performed to assess pro-growth/survival mediators. Data in **A** and **B** show that α-TEA alone suppresses to different degrees RTK/Akt/ERK complex signaling (namely, pHer-1/total Her-1, pHer-2/total Her-2, pIGFR/total IGFR, pAkt, pERK) and Akt/ERK downstream mediators (namely, active form of ER-a and mTOR as well as total protein levels of FASN, c-FLIP and Bcl-2) in comparison to the VEH control in both MCF-7/TamR and MCF-7/Her-2 cells. In contrast, TAM alone either enhances pro-survival signaling mediators or had no major impact on the already high constitutive active basal levels. The impact of the combination α-TEA + TAM treatment is striking. Not only did it overcome any TAM-induced upregulation of these pro-proliferation/pro-survival signal transduction mediators but suppressed them to a much greater extent than even a-TEA was capable of achieving, again highlighting the ability of this dual treatment to dramatically knockdown the functional status of these important pro-proliferation/pro-survival mediators.

siRNA blockage of Akt-1 or c-FLIP (L) enhances the anti-cancer properties of α-TEA

siRNA knock-down of pro-survival Akt-1 induced low levels of apoptosis in MCF-7/TamR cells, but did not induce ER stress biomarker GRP78 (Fig 36). However, siRNAs to Akt-1 and c-FLIP (L) enhanced α-TEA induced increases in ER stress biomarker GRP78 and ER stress mediators pJNK, CHOP and DR5 (L/S), as well as apoptosis detected by PARP cleavage (Fig 5). siRNA to Akt-1 inhibited c-FLIP (L) protein expression, suggesting that Akt-1 is an upstream mediator of c-FLIP (L) (Fig 36).

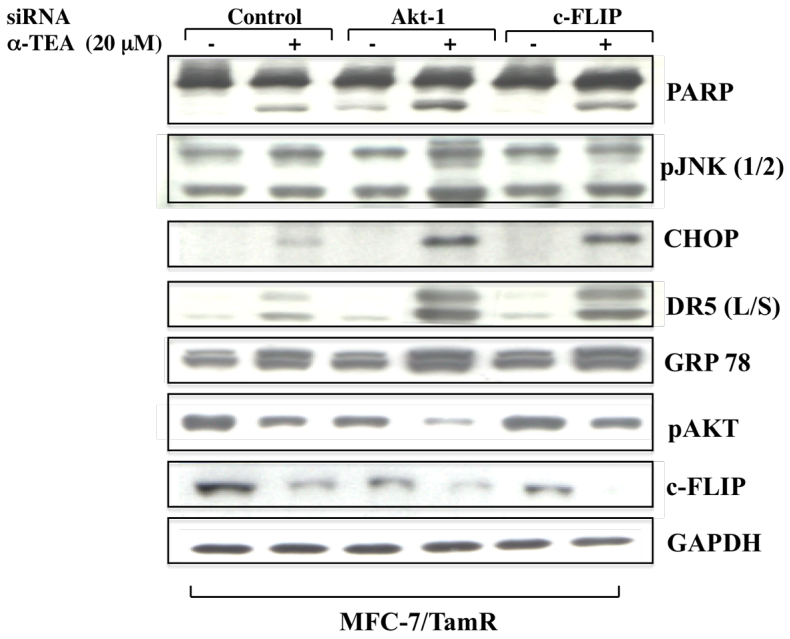


Figure 36: MCF-7/TamR cells were transiently transfected with siRNA to Akt-1 and c-FLIP as well as a nonspecific negative control siRNA (labeled Control) using our standard lab procedures. Transfected cells were cultured for three days prior to collection of whole cell lysates for Western immunoblot analyses. siRNA to Akt-1 and c-FLIP enhanced α-TEA induced apoptosis as indicated by PARP cleavage as well as α-TEA induced upregulation pJNK, CHOP, DR5 and GRP78. siRNA to Akt-1 and c-FLIP further inhibited α-TEA induced downregulation of pAkt and c-FLIP. Data represent at least three separately conducted experiments.

Cholesterol lipid raft contributes to constitutively active pro-survival/anti-apoptotic mediators in TAM resistant cell lines

Cholesterol enriched lipid rafts have important roles in regulating growth factor receptor mediated pro-survival/anti-apoptotic mediators in cancers [Zhuang et al., 2005]. Control TAMR cell lines express high levels of cholesterol enriched lipid rafts, as measured by fluorescent cholesterol lipid raft marker DilC-16 [Corrêa et al., 2009], in comparison with TAM sensitive cell lines (MCF-7/TamS and MCF-7/vector) (Fig 37 A and B). α-TEA inhibited lipid raft (absence of fluorescent marker DilC-16) in both TAMR cells lines (Fig 37 A and B).

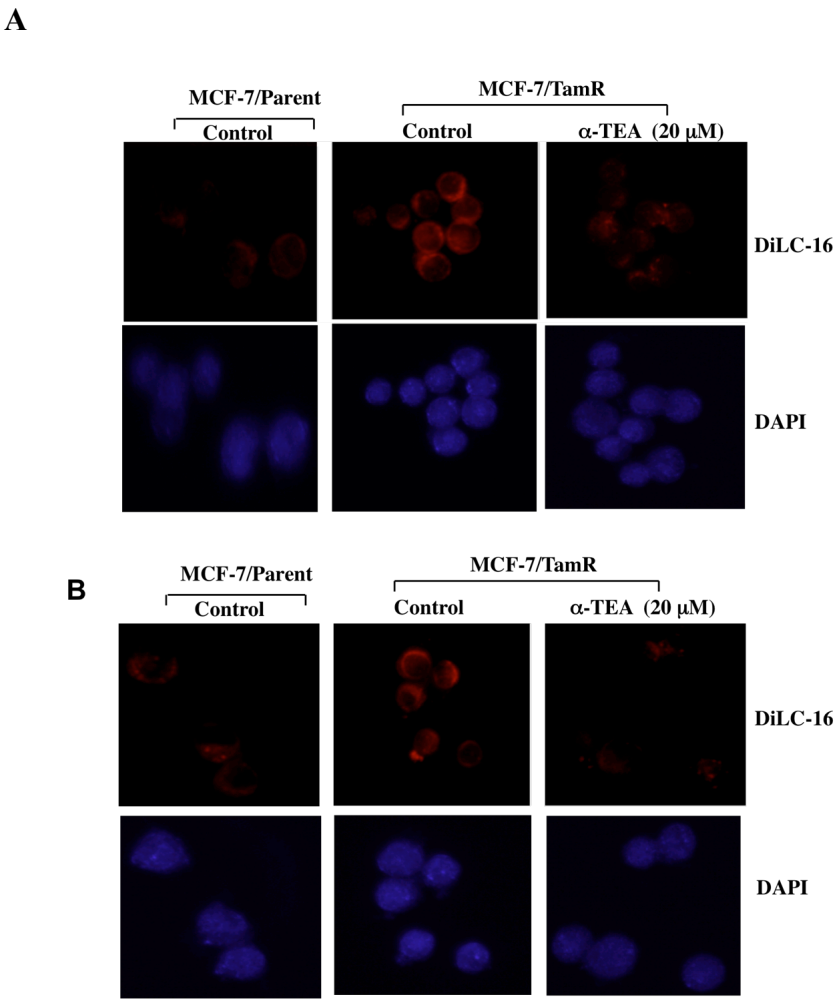
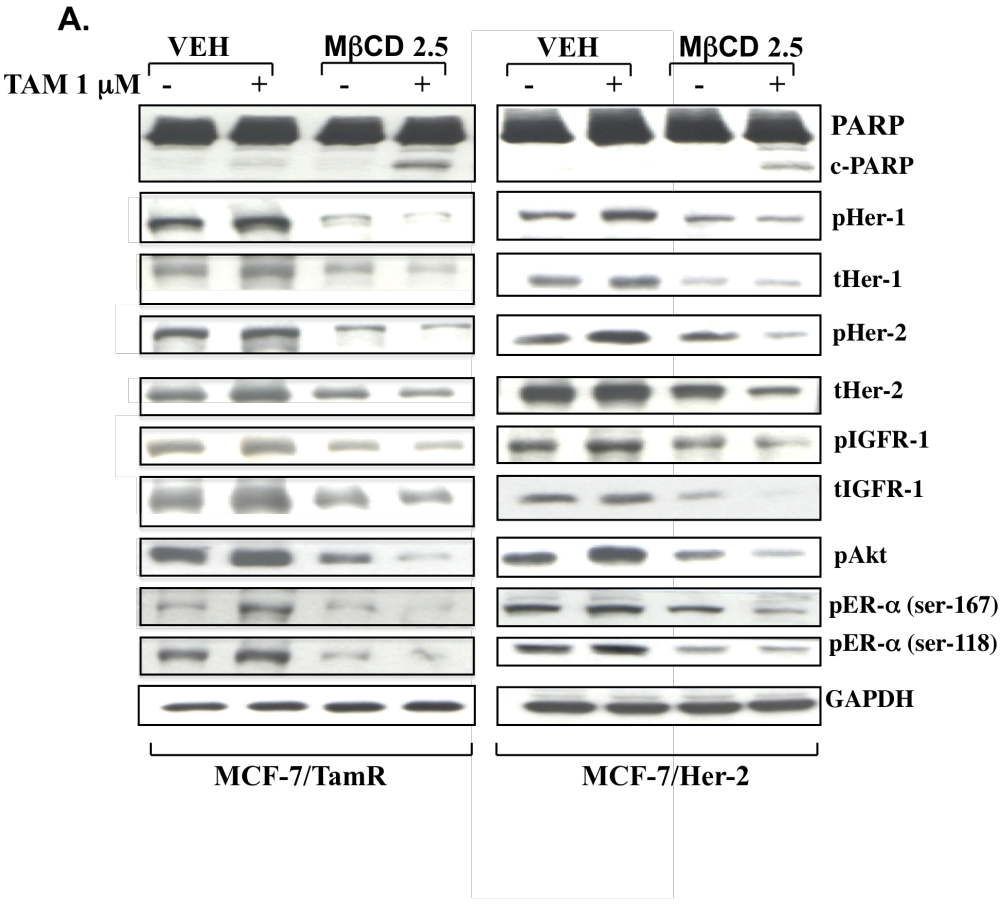


Figure 37: MCF-7/TamR and MCF-7/Her-2 cell surface plasma membranes are highly enriched with cholesterol lipid raft microdomains in comparison to MCF-7/parent and MCF-7/Vector cells. Untreated MCF-7 and MCF-7/TamR and MCF-7/Vector and MCF-7/Her-2 cells were cultured in steroid-depleted media supplemented with E_2 (10^{-9}M) for TAM sensitive cells for one day were stained with lipid raft marker, DiIC-16. A high level of red fluorescent staining is seen in MCF-7/TamR cells in comparison with MCF-7/parent cells (Fig 6A) and α-TEA disrupted cholesterol rich lipid microdomains. Similar disruption of lipid microdomain was observed in MCF-7/Her-2 cells in comparison with MCF-7/Vector cells (Fig 6B). Data are representative of three independent experiments.

Lipid raft disruptor methyl-β-cyclodextrin (MβCD) plus TAM or α-TEA acts cooperatively in TAMR cell lines to induce apoptosis and suppress pro-survival mediators MβCD at 2.5 μM suppresses growth survival mediators in TAM resistant cell lines (Fig 38 A), suggesting that lipid raft participates in growth factor mediated TAM resistance. Furthermore, combination of MβCD + TAM acts cooperatively to as induce apoptosis detected by annexin V/PI staining in TAMR cells (Fig 38 B), suggesting that targeting lipid raft circumvents TAM resistance when treated with TAM.



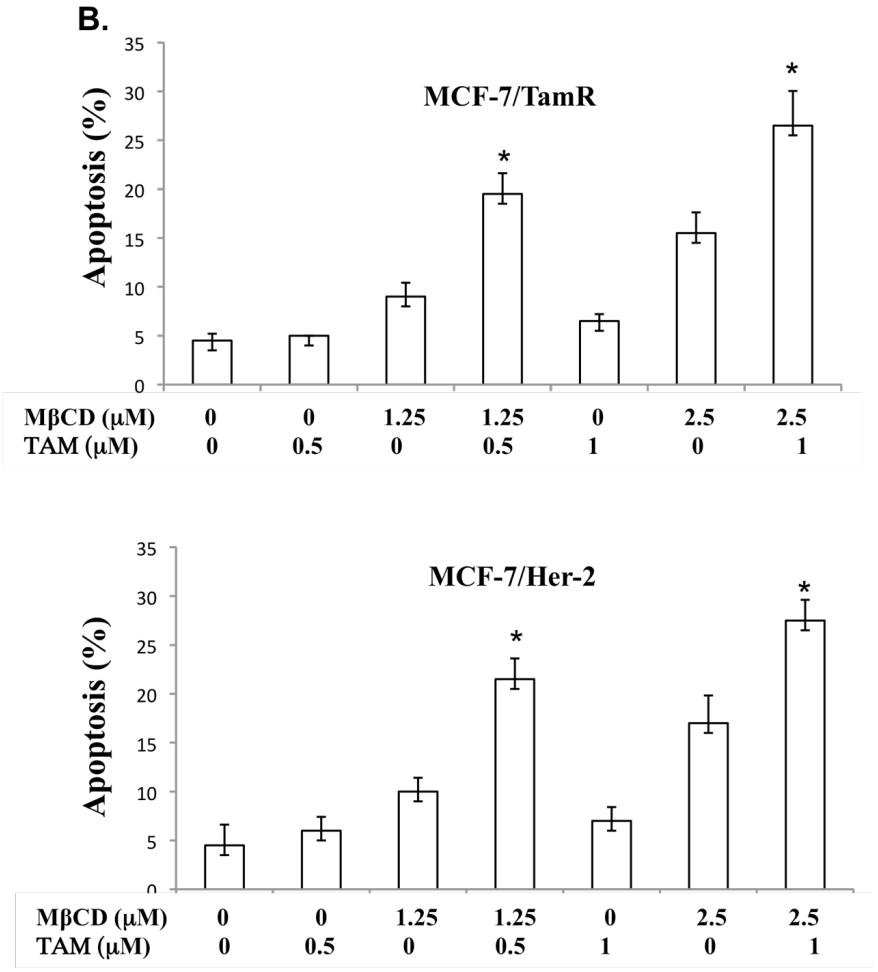
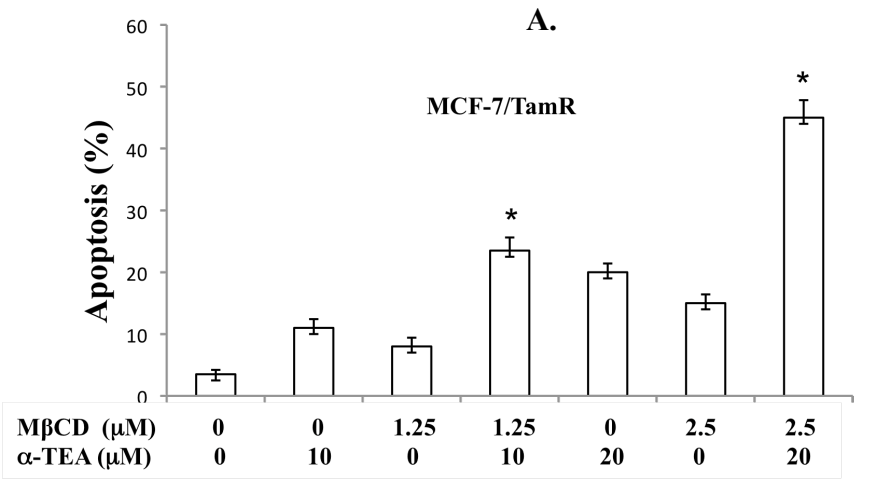
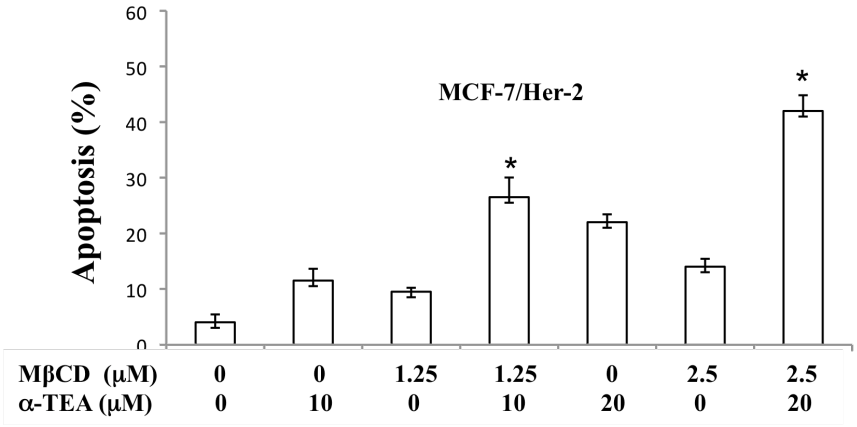


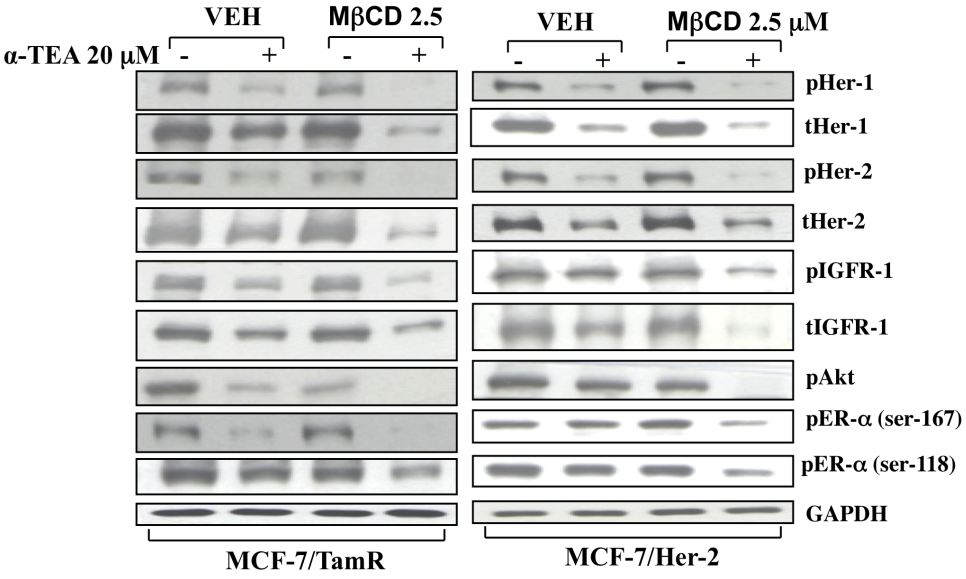
Figure 38: Lipid raft disruptor Methyl-β-cyclodextrin (MβCD) cooperates with TAM to induce apoptosis and suppress pro-survival pathway. **A.** MCF-7/TamR and MCF-7/Her-2 cells were treated with MβCD (1.25 or 2.5 μM), TAM (0.5 or 1 μM) either singly or in combination for 1 day. Apoptosis was determined by FACS analyses after annexin V/PI staining. Data are depicted as mean ± SD of three individual experiments. **B.** MCF-7/TamR and MCF-7/Her-2 cells were treated with 2.5 μM of MβCD, 1 μM of TAM either singly or in combination for 1 day. Western blot analyses show that TAM by itself upregulated the mediators of survival pathway such as pHer-1/total Her-1, pHer-2/total Her-2, pIGFR, pAkt) and Akt/ERK downstream mediators (namely, active form of ER-α). MβCD slightly downregulated these pro-survival mediators but the combination of MβCD and TAM cooperatively inhibited these mediators. Data are representative of three independent experiments.

Furthermore, treatment of both TAMR cell lines with α-TEA alone and more extensively when in combination with MβCD enhanced apoptosis as detected by Annexin V (Fig 39 A) with CI (combination index) at 0.48 and 0.21 for MCF-7/TamR and MCF-7/Her-2 cells, respectively (Table 4), suggesting a synergistic effect on induction of apoptosis. α-TEA cooperated with MβCD to further block the expression of these pro-survival/anti-apoptotic mediators (Fig 39 B) and also enhanced pro-apoptotic mediators of ER stress namely, CHOP, DR5, pJNK and GRP-78 (Fig 39 C).





B.


VEH
MβCD 2.5
VEH
MβCD 2.5 μM
α-TEA 20 μM
- + - +
- + - +
pHer-1
tHer-1
pHer-2
tHer-2
pIGFR-1
tIGFR-1
pAkt
pER-α (ser-167)
pER-α (ser-118)
GAPDH
MCF-7/TamR
MCF-7/Her-2

C.

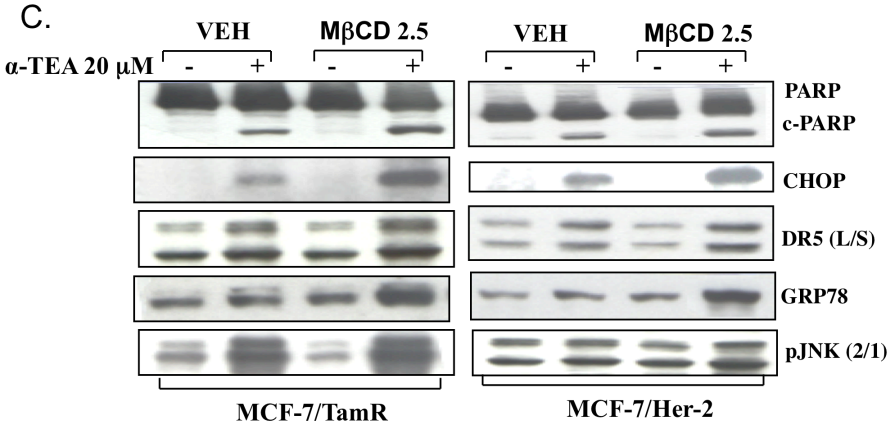

VEH
MβCD 2.5
VEH
MβCD 2.5
α-TEA 20 μM
- + - +
- + - +
PARP
c-PARP
CHOP
DR5 (L/S)
GRP78
pJNK (2/1)
MCF-7/TamR
MCF-7/Her-2

Figure 39: α-TEA suppression of lipid raft contributes to combination circumvention of TAM resistance. **A.** MCF-7/TamR and MCF-7/Her-2 cells were treated with MβCD (1.25 or 2.5 μM), α-TEA (10 or 20 μM) either singly or in combination for 1 day. Apoptosis was determined by FACS analyses after annexin V/PI staining. Data are depicted as mean ± SD of three individual experiments. **B.** MCF-7/TamR and MCF-7/Her-2 cells were treated with 2.5 μM of MβCD, 20 μM of α-TEA either singly or in combination for 1 day. MβCD cooperated with α-TEA to further inhibit the pro-survival mediators. **C.** MβCD not only enhanced α-TEA induced apoptosis as seen by PARP but also further enhanced α-TEA induced ER stress markers namely pJNK, CHOP, DR5 and GRP78 in both MCF-7/TamR and MCF-7/Her-2 cells. Data are representative of three independent experiments.

Table 4 Combination Index (CI) of apoptosis [a]

	α-TEA:MβCD Ratio [b]	CI [c]			[d] Mean ± SD	
		ED50	ED75	ED90		
MCF-7/TamR	8:1	0.6	0.47	0.36	0.48 ± 0.12	Synergism [e]
MCF-7/Her-2	8:1	0.3	0.2	0.14	0.21 ± 0.08	Synergism [e]

[a]MCF-7/TamR and MCF-7/Her-2 breast cancer cells were treated with different concentrations of α-TEA and MβCD alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining / FACS assay as described in Material and Methods.
[b]The ratio for the concentrations that were used in combination treatments were determined from the data in Fig1.
[c]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).
[d]The mean ± SD is calculated from the CI values of ED50, ED75 and ED90.
[e]CI values < 1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

Exogenous cholesterol blocked α-TEA alone and α-TEA + TAM induced apoptosis

Cholesterol, a lipid raft reconstitutor [Pike, 2003], blocked the ability of α-TEA alone and combination of α-TEA + TAM to decrease protein levels of survival mediators (Fig 9). These data show that cholesterol can block the anti-cancer actions of α-TEA and α-TEA plus TAM combination.

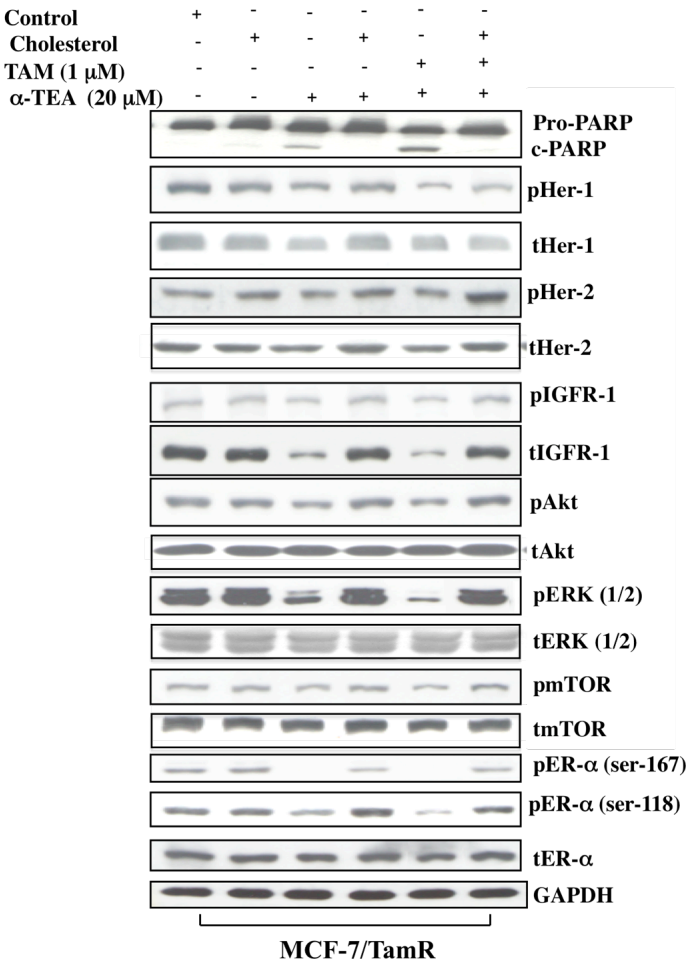


Figure 40: α-TEA suppression of lipid raft contributes to combination circumvention of TAM resistance. **A** MCF-7/TAMR cells were pre-treated with water-soluble cholesterol at 50 mg/ml (Sigma) for 2 hrs followed by culturing with α-TEA at 20 μM or α-TEA + TAM (20 μM of α-TEA + 1 μM TAM) for 1 day. Western blot analyses were performed to detect pro-survival mediators. Cholesterol, a lipid raft reconstitutor, blocks α-TEA and combination of α-TEA + TAM induced apoptosis detected by PARP cleavage as well as suppression of survival pathway.

5.4. Discussion

Acquired and de novo TAM resistance are major barriers for successful application of TAM in the clinic. Data reported here show that α-TEA, a small bioactive lipid, possesses the ability to restore TAM sensitivity when combined with TAM in both acquired and de novo TAM resistant breast cancer cell lines. Novel findings in this study are: (i) Combination of α-TEA + TAM acts cooperatively to induce ER stress mediated JNK/CHOP/DR5 pathway and ER stress mediated apoptosis, (ii) Combination of α-TEA + TAM acts cooperatively to suppress pro-survival/anti-apoptotic pathways, (iii) Inhibition of Akt-1 and c-FLIP pro-survival/anti-apoptotic factors enhances α-TEA induced ER stress and apoptosis, (iv) Targeting lipid raft is a potential strategy to circumvent TAM resistance when combined with TAM and is involved in combination of α-TEA + TAM circumvention of TAM resistance. As a vitamin E derivative, α-TEA exhibits selective toxicity to cancer cells, but not to normal cells and tissues [Latimer et al., 2009]. Therefore, combination of α-TEA + TAM shows potential as a novel combination to prevent and circumvent TAM resistance in human ER-positive breast cancer.

It is well established that TAM resistance is caused by over expression of receptor tyrosine kinase (RTK) proteins such as Her-1 and Her-2, which enhances their cross-talk with membrane-associated estrogen receptor (mER) resulting in cell

proliferation and survival via their downstream mediators, such as Akt, ERK, mTOR, in which TAM acts as agonist to stimulate the growth signaling pathway [Massarweh et al., 2006; Arpino et al., 2008]. Targeting the components in this highly amplified proliferation/survival mediators, such as Her-1, Her-2, Akt and mTOR using chemical inhibitors or antibodies has been reported to circumvent TAM resistance [Ghayad et al., 2010, deGraffenried et al., 2004; Leary et al., 2010]. Here we show that TAM alone stimulates growth factor expression, but synergizes with α-TEA to suppress proliferation/survival mediators in TAMR cells suggest that in the combination of α-TEA plus TAM, TAM is converted from an agonist to an antagonist, thereby amplifying proliferation/survival factors signatures of TAMR cells. Thus, our data show that α-TEA as an inhibitor of proliferation/survival mediators converts TAMR cells to TAMS cells.

In addition to α-TEA's inhibitory role in proliferation/survival pathways, α-TEA possesses potent pro-apoptotic activity, which growth factor inhibitors used in the clinic lack. It appears that α-TEA's dual role of down-regulation of proliferation/survival mediators and up-regulation of death signaling mediators are both important for circumventing TAM resistance. α-TEA induces apoptosis via activation of ER stress mediated JNK/CHOP/DR5 pro-apoptotic pathway [Tiwary et al. 2010]. Although TAM induces apoptosis in TAM sensitive cells it did not induce apoptosis in TAM resistant cells even at off target levels (10 μM) (data not shown). However, TAM synergizes with α-TEA via promotion of not

only caspase-8 and 9 apoptotic cascade but also ER stress mediated JNK/CHOP/DR5.

It is well established that targeting growth factor signaling causes apoptosis in growth factor addictive cell types and promotes apoptosis induced by pro-apoptotic agents [Meng et al., 2010; McCubrey et al., 2006]. Akt is an anti-apoptotic factor and exerts its survival role via a diverse array of substrates which control key cellular processes, including apoptosis [Chang et al., 2003]. A major downstream substrate of Akt is the serine/threonine kinase mTOR. Akt can directly phosphorylate mTOR at ser-2448 and activate it, as well as cause indirect activation of mTOR by phosphorylating and inactivating tuberous sclerosis complex 2 (TSC2), also called tuberin). The Raptor-mTOR-complex signals to its downstream effectors S6 kinase/ribosomal protein S6 (p70S6K) and the eIF4E binding protein (p4E-BP1**)** to control transcription and translation, which selectively regulates multiple proteins that control cell cycle and apoptosis [Gibbons et al., 2009]. Additionally, Akt can directly regulate apoptosis by phosphorylating and inactivating pro-apoptotic proteins such as Bad and caspase-9 [Datta et al., 1997; Cardone et al., 1998; Mabuchi et al, 2002]. Downregulation of these Akt mediated anti-apoptotic factors or upregulation of these Akt suppressed pro-apoptotic factors can contribute to promotion of α-TEA induced mitochondria dependent apoptotic cascade.

How low dose of TAM plus α-TEA synergize to induce ER stress is not fully understood. One possibility is that there may be cross talk between ER stress and growth factor pathways. Previous data show that downregulation of c-FLIP can enhance α-TEA induced ER stress via activation of caspase-8 by the evidence showing that caspase-8 is involved in α-TEA induced ER stress [Tiwary et al., 2010]. Since Akt can be upstream mediators of c-FLIP, we investigated if suppression of Akt and c-FLIP is involved in α-TEA-induced ER stress. As expected, data show that inhibition of Akt-1 blocks c-FLIP protein expression, suggesting that Akt is upstream mediator of c-FLIP. Inhibition of both Akt-1 and c-FLIP enhanced α-TEA induced ER stress, evidenced by increased ER stress marker, GRP78 and ER stress mediated JNK/CHOP/DR5 cascade. Although downregulation of c-FLIP was identified as a downstream event of ER stress in α-TEA induced apoptosis in MCF-7 breast cancer cells [Tiwary et al., 2010], our present data show that c-FLIP expression is mediated in part by Akt. Here, for the first time, we demonstrate that suppression of Akt can enhance α-TEA mediated ER stress. It will be interesting to see if these mediators are implicated for other ER stress inducers induced ER stress. Based on our previous data, we conclude that c-FLIP enhances α-TEA induced ER stress via activation of caspase-8, leading to promotion of caspase-8/ER stress positive loop. Inhibition of Akt enhances α-TEA induced ER stress via Akt inhibition of c-FLIP.

TAM has been reported to induce GRP78, an ER stress marker in TAMS cells at high doses [Scriven et al., 2009]. The induction of ER stress was not observed in TAMS cells with siRNA knock down of ER-α, or treatment with 5 μM TAM or lower doses (data not shown). Furthermore, ER stress was not observed in TAMR cells sensitized to TAM treatments by siRNA knockdown of Akt plus treatment with TAM (1 μM), although growth factor signaling mediators were significantly suppressed by this combination (data not shown). These data show that sensitizing TAMR cells to TAM does not explain the cooperative actions of α-TEA plus TAM on induction of ER stress. Furthermore, data show that α-TEA is a unique bioactive lipid that can circumvent TAMR when combined with TAM not only via converting TAM from an agonist to an antagonist but also via triggering apoptosis.

Cholesterol lipid raft microdomains have been shown to provide a platform for crosstalk among growth factor receptors and their downstream mediators leading to enhanced proliferation/survival [Marquez et al., 2006; Patra, 2008]. Cholesterol-rich lipid rafts have been reported to participate in crosstalk between mER and growth factor receptors [Pike, 2005]. Therefore, targeting cholesterol lipid raft may be a promising regimen to inhibit cell proliferation and cell survival. Our data show that TAM resistant cells express high levels of cholesterol enriched lipid raft, detected by lipid raft marker. MβCD, a cholesterol disruptor suppresses TAMR pro-survival/an-apoptotic signature, suggesting that

cholesterol lipid rafts contribute to the constitutively activated proliferation/survival mediators in TAM resistant cells. Furthermore, data show that combination of MβCD + TAM act cooperatively to induce apoptosis and suppress pro-survival mediators, suggesting that the combination of MβCD + TAM circumvents TAM resistance. Data show that α-TEA disrupted cholesterol lipid raft as detected by loss of DilC-16 staining (lipid raft marker), and that cholesterol blocked α-TEA induced suppression of growth signaling pathway, and that combination of MβCD + α-TEA cooperatively acts to induce ER stress and suppress survival pathway, as well as induced apoptosis, suggest that α-TEA suppresses growth signaling pathway, at least partially, via disruption of cholesterol lipid rafts. α-TEA disruption of lipid raft detected by lipid raft marker has been found in other cell types (data not shown), suggesting that α-TEA disruption of cholesterol lipid raft is an initial event in suppression of growth factor signaling pathways. How α-TEA disrupts cholesterol lipid raft is not known. Possibilities include: (i) α-TEA activation of ceramide lipid raft replaces cholesterol lipid raft, and (ii) α-TEA, a detergent-like bioactive lipid, acts as direct disruptor of cholesterol lipid raft. These possibilities are under investigation.

In summary, α-TEA functions as an endoplasmic reticulum inducer and cholesterol lipid raft disrupter to circumvent TAM resistance when treated with

TAM via activation of ER stress mediated pro-apoptotic mediators and suppression of highly amplified survival mediators (figure 41). Data presented here demonstrate the potential of α-TEA + TAM as a novel combination for prevention and circumvention of TAM resistance in ER-positive, TAM resistant human breast cancers and provides new knowledge about mechanisms of endocrine therapy resistance that may serve as future targets for circumvention of TAM resistance.

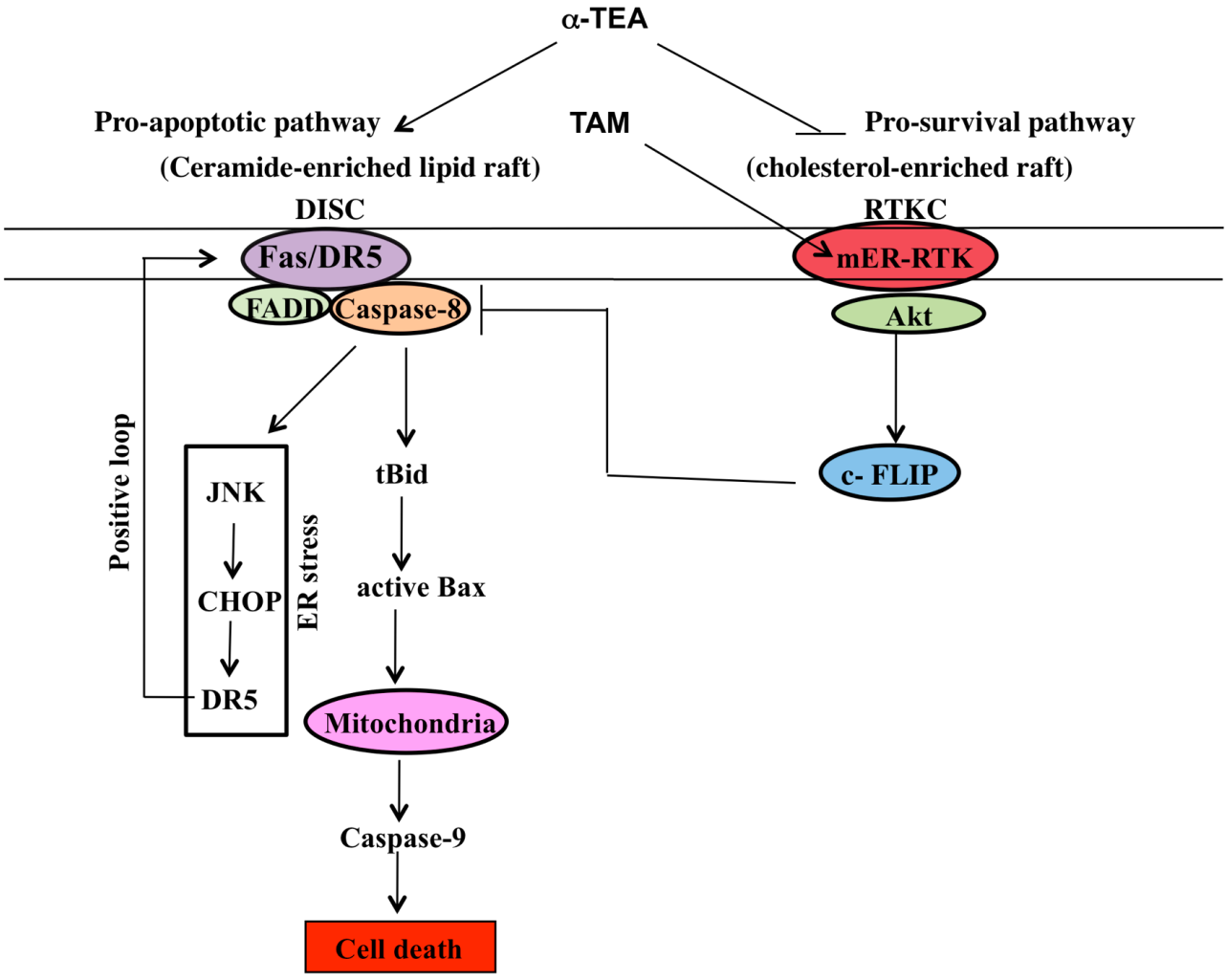


Figure 41: Proposed signaling pathways modulated by α-TEA and TAM in circumvention of TAMR. Based on our published and present data a schematic diagram of the actions of α-TEA and TAM alone and in combination on pro-apoptotic and pro-survival signaling pathways in TAMR cells are depicted in Figure 10. (i) Pro-apoptotic pathway impacted by α-TEA: α-TEA triggers Fas/DR5 extrinsic death receptor-mediated apoptotic pathways via the following cascade of events. Activation of ASMase, leading to increase in ceramide-rich lipid raft microdomains which provides platforms for death-inducing signal complex (DISC) formation and activation, thereby triggering caspase-8-mediated mitochondria-dependent apoptotic cascade (caspase-8→Bid→truncated (active) tBid→Bax→mitochondria→caspase-9→apoptosis), and caspase-8-mediated endoplasmic reticulum stress-dependent upregulation of a JNK/CHOP/DR5 positive

feedback loop to amplify levels of DR5 and subsequent caspase-8-mediated mitochondria-dependent apoptotic cascade. (ii) Pro-survival pathway impacted by α-TEA: α-TEA suppresses total and phosphoprotein levels of RTKs (Her-1, Her-2 and IGFR), decreases levels of phospho-Akt (pAkt), mTOR (pmTOR), ERK (pERK1/2) and ER-α (ser-118 and 167) and levels of c-FLIP via disrupting cholesterol lipid raft. Suppression of survival/anti-apoptotic factors and activation of pro-apoptotic factors reveal the dual actions of α-TEA in TAMR cells. (iii) Pro-survival pathways impacted by TAM: In contrast to α-TEA, TAM treatment of TAMR cells enhances pro-survival pathways via crosstalk between membrane associated ER (mER) and RTK. [It is important to note that α-TEA converts TAM's pro-survival (agonistic) actions to anti-pro-survival (antagonistic) effect when treated with TAM]. (iv) Combination of α-TEA + TAM cooperatively act to induce apoptosis via ER stress mediated pro-apoptotic pathway: Suppression f the pro-survival pathway in TAMR cells by treatment with the combination of α-TEA + TAM act cooperatively to induce JNK/CHOP/DR5 mediated caspase-8 dependent apoptosis by different mechanisms (please see discussion) including suppression of anti-apoptotic mediator c-FLIP. As reported previously by our lab, downregulation of c-FLIP leads to enhancement of a caspase-8/ER stress/JNK/CHOP/DR5 pro-apoptotic positive-acting amplification loop. In summary, data presented here show that the dual actions of α-TEA plus TAM combination have high chemotherapeutic potential in TAMR cancers via cooperatively inducing TAMR cells to undergo apoptosis via circumventing TAM resistance.

Chapter 6. α-TEA targets FASN to induce apoptosis in tamoxifen resistance breast cancer

Introduction:

It is well established that estrogen (E2) is involved in the pathogenesis of breast carcinoma, and that estrogen sustains growth of estrogen-responsive breast cancers [Russo et al., 1998]. Approximately 70% of all breast tumors rely on estrogen for survival and are referred as estrogen dependent. Tamoxifen (TAM), first line endocrine therapy (ET), is highly effective for ER responsive breast cancers [Lewis et al., 2005]. Unfortunately, 40-50% of all ER+ breast cancer patients will either not respond to first line TAM treatment or after initial successful response, will suffer cancer relapse [Susan G. Komen for the Cure: Breast Cancer Facts, 2009]. These findings strongly suggest that *de novo* or acquired TAM resistance (TAMR) occurs in these patients. TAM treatment failure occurs when tumors become insensitive to TAM or become stimulated rather than inhibited by TAM. There is an urgent need to better understand TAMR and to develop novel regimens to prevent/circumvent TAMR.

Several mechanisms of TAMR have been identified for both de novo and acquired TAM resistance [Musgrove et al., 2009; Hurvitz et al., 2008]. Of the many possibilities, aberrant over-expression of growth factor signaling pathways are implicated as important contributors to both acquired and de novo TAMR [Arpino et al., 2008]. It is well established that in ER^+ TAMR cells, over-

expressed receptor tyrosine kinases (RTKs) Her-1 and Her-2 and downstream mediators Akt and ERK, as well as ER-α promote E2 independent cell proliferation and inhibit apoptosis [Massarweh et al., 2006]. Crosstalk among pro-survival/anti-apoptotic factors, RTK and membrane associated ER-α (mER-α), Her-1/Her-2 and IGF1R, and between nuclear ER-and Her-1/Her-2, are involved in maintaining TAMR [Knowlden et al., 2005].

Fatty Acid Synthase (FASN), the enzyme that catalyzes the terminal steps in de novo biosynthesis of long-chain fatty acids, is a tumor-associated gene, is highly expressed tumors, and minimally expressed in most normal human tissues except liver and fat tissues [Milgraum et al., 1997]. FASN has been reported to function differently in non-cancer versus cancer cells. FASN has a key role in the conversion of carbohydrates to lipids in liver and adipose tissues, and produces essential lipid components for membrane function and for endoplasmic reticulum homeostasis of cancer cells [Wakil et al., 1989]. Expression of the FASN gene in tumors has been reported to be correlated with transformation and tumor promotion/ progression [Kuhajda et al., 2000]. Inhibition of FASN has been shown to suppress tumor growth, sensitize cells to chemotherapeutic drugs and induce cell death by apoptosis [Mashima et al., 2009*; Pizer et al., 1996*]. FASN is an ideal anti-cancer target since tumor cells are sensitive to reduced FASN levels.

FASN is regulated by sterol receptor element binding protein (SREBP-1), a key lipogenic transcription factor, by stimulating the transcriptional activity of the FASN promoter harboring the complex SREBP-1 binding sites [Boizard et al., 1998]. It has been reported that well-characterized cancer-related growth factors, hormones, and oncogenes activate the SREBP-1 pathway and lead to increased FASN gene expression [Pizer et al., 1998; Kumar-Sinha et al., 200; Swinnen et al., 2000]**.** SREBP-1 is regulated by Akt and ERK via SREBP-1 up-regulation and nuclear maturation [Mashima et al., 2009]. Besides mediating endogenous fatty acid metabolism, FASN is involved in gene regulation. FASN has been reported to upregulate Her-1 and Her-2 mRNA and protein levels in tumor cells [Vazquez-Martin et al., 2008], thereby promoting tumor growth. Thus, inhibition of FASN possesses dual anti-cancer actions: activation of pro-apoptotic and suppression of anti-apoptotic mediators.

RRR-α-tocopherol ether-linked acetic acid analog (α-TEA), a unique small active lipid, has been shown to possess nontoxic anticancer properties both *in vitro* and *in vivo* [Kline et al., 2007; Latimer et al., 2009]. Molecular mechanistic data show that the anti-cancer actions of α-TEA are by dual functions: (i) activation of pro-apoptotic pathway mediators including Fas/Fas L [Jia et al., 2008], ER stress mediated JNK/CHOP/DR5 [Tiwary et al., 2010], p73/NOXA [Wang et al., 2008], leading to caspase-8 and mitochondria dependent

apoptosis and (ii) suppression of pro-survival/anti-apoptotic factors such as Her-1, Her-2, pAkt/pmTOR, pERK, c-FLIP, Bcl-2 and Survivin [Shun et al., 2010].

In this study, we used acquired and de novo TAMR cell lines; MCF-7/TamR and MCF-7/Her-2, respectively, to determine: (i) if FASN is involved in maintaining TAMR, (ii) if knockdown of FASN induces apoptosis in TAMR cells and (iii) if α-TEA suppresses FASN expression, and combination α-TEA plus FASN inhibitor C75 provides a novel regimen to treat TAMR breast cancer cells.

6.2. Material and Methods:

Chemicals

α-TEA was prepared in our lab as described previously in chapter 5. TAM was purchased from Calbiochem (La Jolla, CA). C75 (FASN inhibitor) was purchased from Sigma (St. Louis, MO).

Cell Culture

The following human breast cancer cells were used in this study: isogenic pair of MCF-7/S parental and MCF-7/TAMR, isogenic pair of MCF-7S vector and MCF-7Her-2 cell lines, and MCF-7/Akt. MCF-7/TAMR and MCF-7S parental, and MCF-7-Akt cell lines were kind gifts from Dr. Linda deGraffenried. MCF7/neo (referred to as MCF-7/vector) and MCF-7/Her2-18 (referred to as MCF-7/Her2) cell lines were a kind gift from Dr. Mien-Chie Hung. Cell lines were cultured as described previously in chapter 5.

Western Blot Analyses

Whole cell protein extracts were prepared and western blot analyses were conducted as described previously. Proteins at 20-50 mg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), CHOP, GRP-78, and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA), Caspase-8, Caspase-9, DR5, phospho-IGF1R, IGF1R, phospho-Her-1, Her-1, phospho-Her-2,

Her-2, FASN, phospho-ERα (ser-118), phospho-ERα (ser-167) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA).

RNA Interference

A scrambled RNA duplex purchased from Ambion (Austin, TX) that does not target any of the known genes was used as the nonspecific negative control for siRNA knock down studies (referred to as control siRNA). Transfection of TamR cells with siRNA to JNK, DR5, CHOP, FASN or control (Ambion, Austin, TX) was performed in 100 mm cell culture dishes at a density of 2 x 10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After one day of exposure to transfection conditions, the cells were re-cultured in 100 mm dish at 2 x 10^6 cells/dish and incubated for one day followed by treatments.

Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assay (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Annexin V-FITC/PI assay measures 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 DNA binding dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in late stages of apoptosis. 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.

Statistical Analyses

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey test for comparison of more than two treatments or a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p<0.05$.

6.3. Results

FASN is highly expressed and stimulated by TAM in TAMR cell lines

In comparison with TAMS parental and TAMS vector cells, FASN is highly expressed in both TAM resistant cell lines. TAM inhibits FASN protein expression in both TAMS cell lines and induces FASN protein expression in both TAMR cell lines (Fig 42 A). TAM-induced FASN in the TAMR cell lines is correlated with TAM-enhanced expression of cellular proliferation mediators Her-1, Her-2, pER-α (ser-167) and pAkt in both TAMR cell lines (Fig 42 B). In contrast, in TAM-treated TAMS cell lines, Her-1 and Her-2 were not expressed, and pER-α (ser 167) and pAkt phosphoprotein levels were decreased (Fig 42 B). These data suggest that FASN is involved in tumor cell survival and TAM resistance.

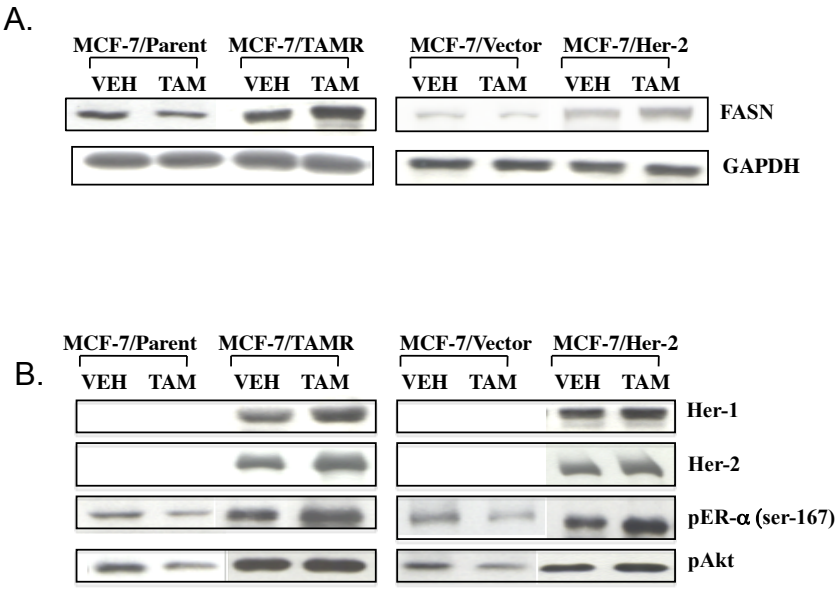
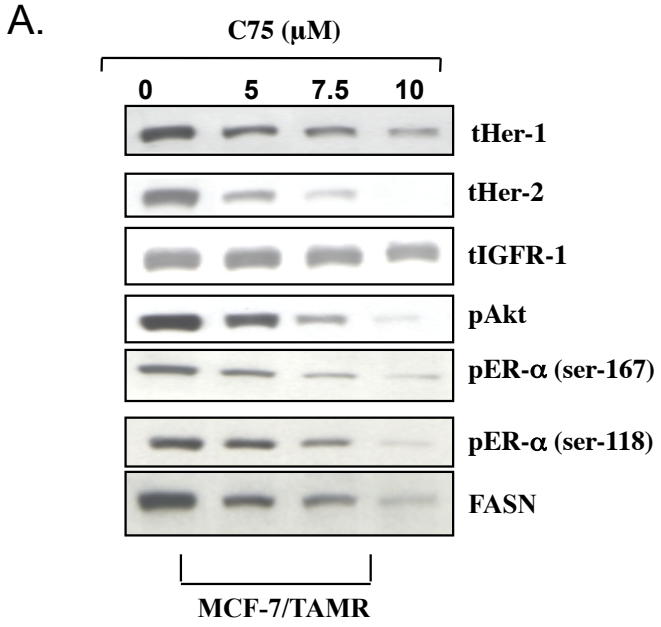


Figure 42: FASN is highly expressed in TAM resistant cells. Western blot analyses were performed on whole cell lysates from MCF-7/parent and MCF-7/TamR and MCF-7/Vector and MCF-7/Her-2 cells cultured for three days in steroid-depleted media prior to treatment with TAM (1 μM) or vehicle (VEH) with E_2 (10^{-9}M) for TAM sensitive cells, for two days. FASN proteins levels (**A**), and Her-1, Her-2, p-ERα (ser-167) and pAkt protein levels were determined by western immunoblot analyses (**B**). Data for A and B are representative of three separately conducted experiments.

Inhibition of FASN suppresses TAMR growth factor /pro-survival signature profile

To confirm if FASN is involved in TAMR resistance, the impact of knock down of FASN using chemical inhibitor (C75) and siRNA on TAM proliferation/survival mediators in both TAMR cell lines was examined. FASN inhibitor (C-75) and siRNA suppressed total protein levels of Her-1, Her-2, and IGF1R, and suppressed pAkt and pER-α (ser-167 and ser-118) (Fig 43 A and B). Reduced levels of FASN protein show efficacy of the two knock down agents. These data clearly implicate activated FASN as playing a role in TAM resistance.



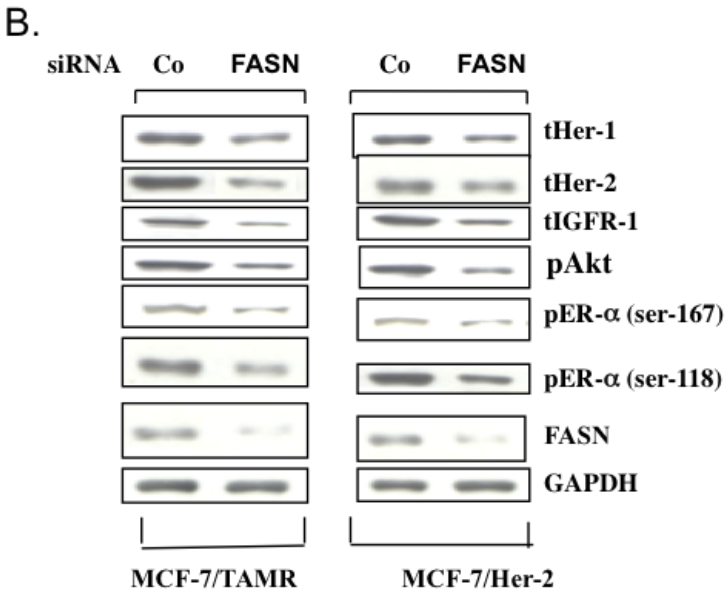


Figure 43: Inhibition of FASN suppresses pro-survival mediators. **A.** MCF-7/TamR and MCF-7/Her-2 cells were treated with FASN inhibitor C75 at 5, 7.5 and 10 μM for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of total Her-1, Her-2 and IGFR-1 protein levels, pAkt, pERα (ser-167 and ser-118) and FASN. GAPDH served as lane controls. **B.** MCF-7/TamR and MCF-7/Her-2 cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of total Her-1, Her-2 and IGFR-1 protein levels, pAkt, pERα (ser-167 and ser-118) and FASN. GAPDH served as lane controls. Data are representative of three separately conducted experiments.

Akt1 is involved in FASN protein expression and participates in regulating growth factor receptors Her-1, Her-2 and IGFR as well as pER-α (ser-167 and 118)

Next, we investigated if FASN is regulated by Akt in the TAM resistant cell lines. siRNA to Akt-1 reduced pAkt,1 FASN, IGF1R, Her-1, Her-2, and pERα (ser-167 and 118) protein levels in both TAM resistant cell lines (Fig 44 A). Additional support for Akt involvement in TAM resistance comes from studies using MCF-7 cells stably transfected with myristoylated, constitutively active Akt1 plasmid (MCF-7/Akt). MCF-7/Akt cells expressed elevated levels of pAkt and total Akt, expressed higher levels of Her-1, Her-2, IGF1R, FASN, pERα at ser-167 and ser-118 in comparison to the non-myristoylated vector control MCF-7 cells (Fig 44 B). These data suggest crosstalk between Akt and proliferation/survival mediators.

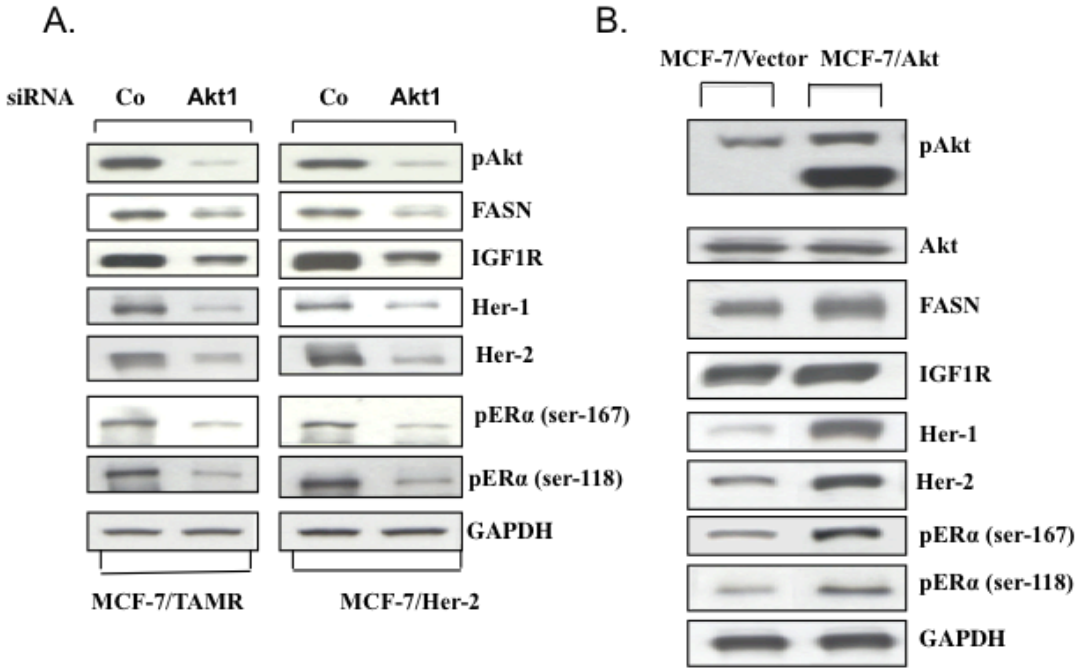


Figure 44: Role of Akt1 in FASN and FASN regulated growth factor protein expression. **A.** MCF-7/TamR and MCF-7/Her-2 cells were transiently transfected with siRNA to either Akt-1 or a nonspecific negative control siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of pAkt, FASN, IGF1R, Her-1, Her-2, pERα (ser-167 and ser-118) and GAPDH. **B.** Whole cell lysates from MCF-7 cells stably transfected with plasmid for the myristoylated, constitutively active Akt1 (MCF-7/Akt) and MCF-7/Vector cells were analyzed by Western immunoblot for levels of pAkt, Akt, FASN, IGF1R, Her-1, Her-2, pERα (ser-167and ser-118) and GAPDH. Data represent three separately conducted experiments.

Interdependent network of proliferation/survival mediators contribute to TAM resistance

To further investigate interactions among growth factor/survival mediators in TAMR cells, ER-α, Her-1, Her-2, and IGF1R were knocked down using siRNA, followed by determining impact on growth factor and pro-survival (FASN) elements. Surprisingly, siRNA knockdown of any single element resulted in suppression of all other members in this inter-related growth factor/survival mediating complex (Fig 45). These data suggest crosstalk among growth factors and that FASN is critical for maintaining TAM resistance.

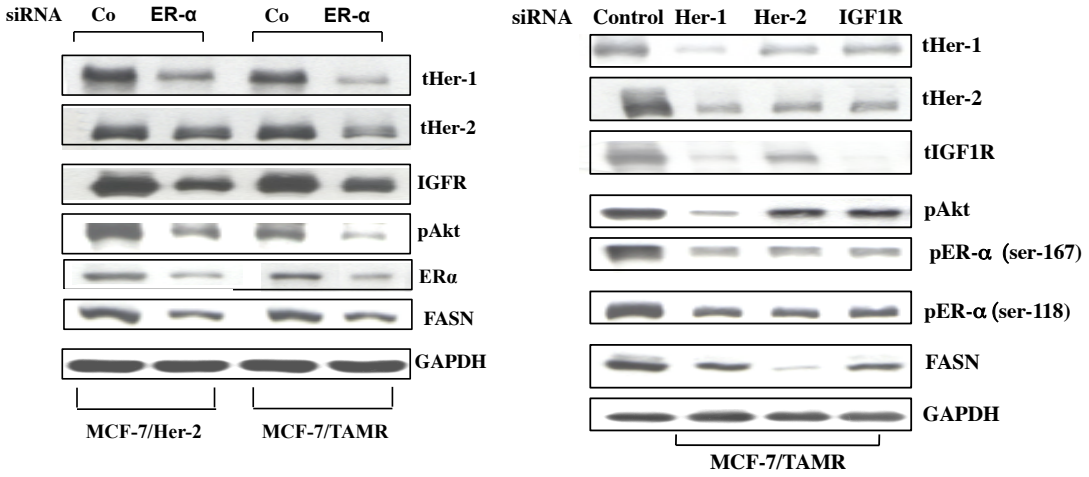
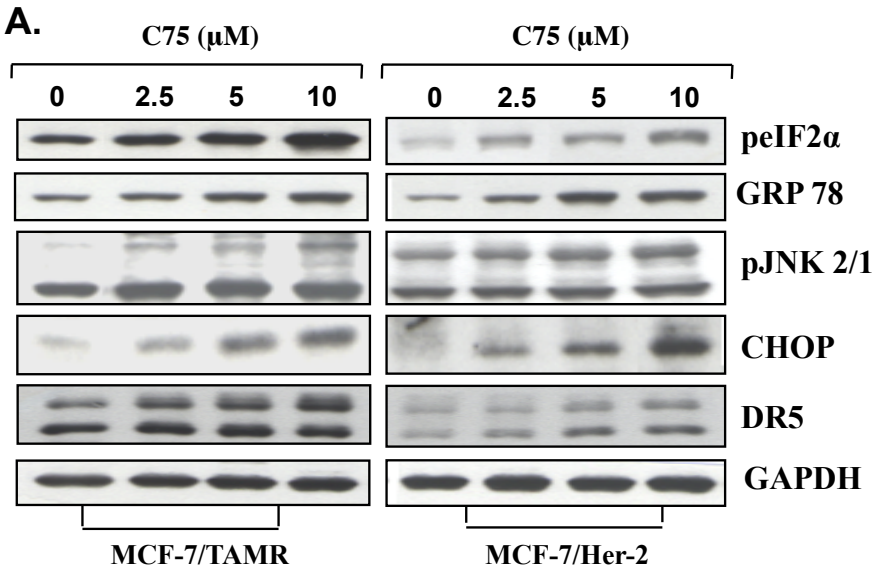


Figure 45: Positive feedback loops and cross talk among growth factors in TAMR cells. MCF-7/TamR and MCF-7/Her-2 cells were transiently transfected with siRNA to Her-1, Her-2, IGF1R or ERα or a nonspecific negative control siRNA for 1 day prior to collection of whole cell lysates for western immunoblot analyses of total (t) Her-1, Her-2, and IGF1R as well as pAkt, pERα (ser-167 and ser-118) FASN and GAPDH. Data are representative of three separately conducted experiments.

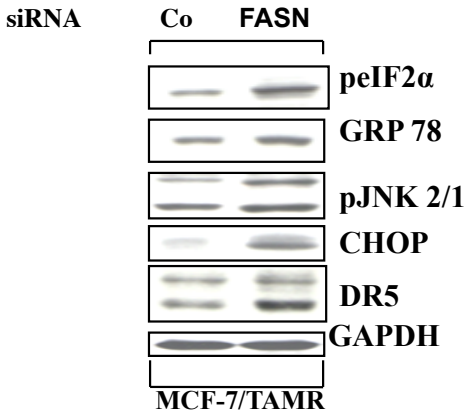
Inhibition of FASN induces apoptosis in TAMR cell lines via endoplasmic reticulum (ER) stress

Western blot data show that FASN C-75 inhibitor or siRNA induces increased levels of ER stress markers peIF2α and GRP78 as well as pro-apoptotic factors pJNK2/1, CHOP, and DR5 (Fig 46 A and B). Since JNK/CHOP/DR5 have been reported to be coupled with ER stress [Tiwary et al., 2010], knockdown assays were conducted to determine if JNK/CHOP/DR5 were involved in FASN inhibition of apoptosis. siRNAs to CHOP, DR5, or JNK blocked FASN C75 inhibitor induced apoptosis (Fig 46 C). These data, for the first time, demonstrated that inhibition of FASN induces apoptosis via ER stress mediated JNK/CHOP/DR5 pathway.

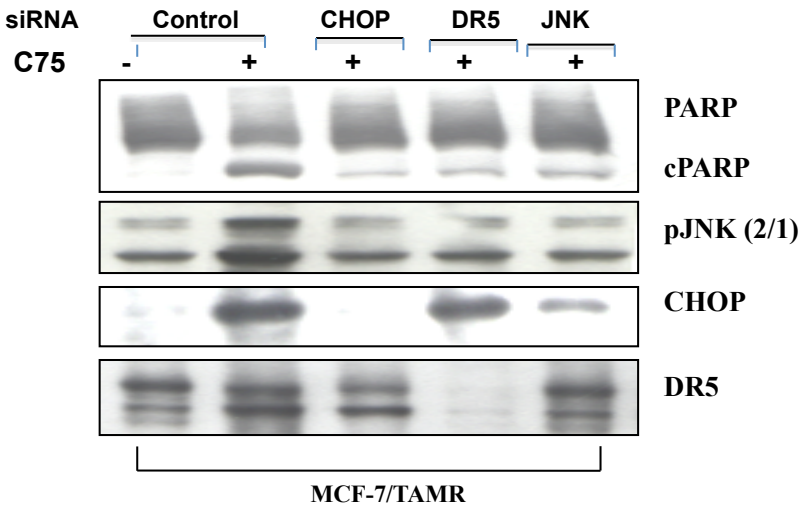
Furthermore, knockdown of FASN using FASN inhibitor (C-75) or siRNA causes TAMR as well as TAMS cells to undergo apoptosis (Fig 46 D and E).



B.


siRNA
Co
FASN
peIF2α
GRP 78
pJNK 2/1
CHOP
DR5
GAPDH
MCF-7/TAMR

C.


siRNA
Control
CHOP
DR5
JNK
C75
-
+
+
+
+
PARP
cPARP
pJNK (2/1)
CHOP
DR5
MCF-7/TAMR

D.

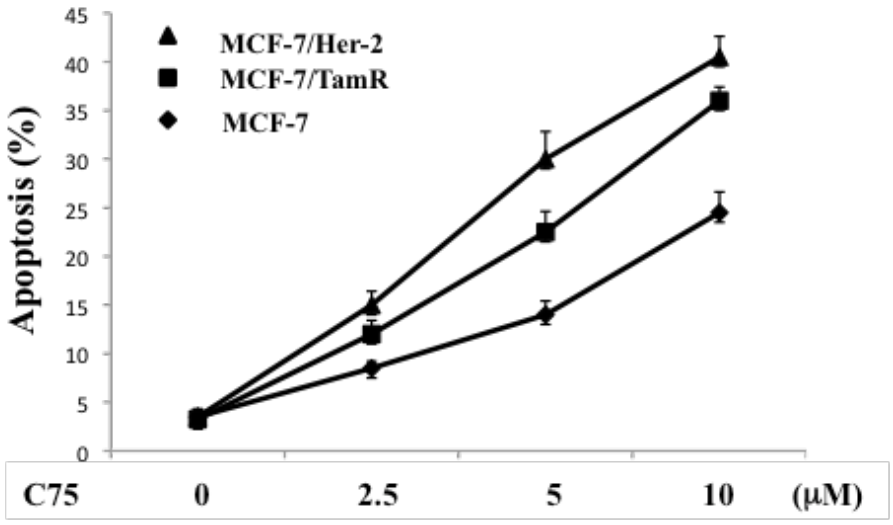
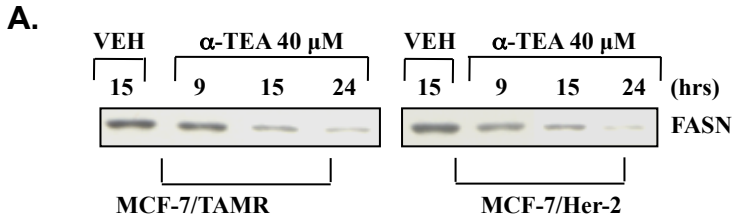


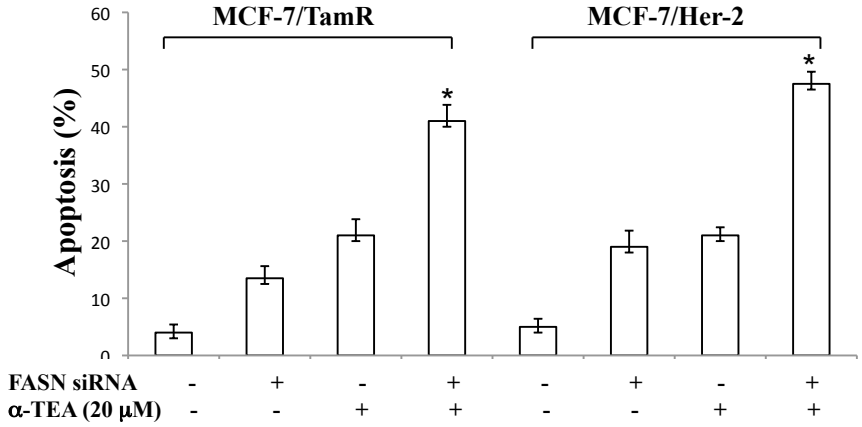
Figure 46: Inhibition of FASN induces endoplasmic reticulum (ER) stress and apoptosis. **A.** MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 at 2.5, 5 and 10 μM for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of peIF2α, GRP-78, pJNK2/1, CHOP, DR5 and GAPDH. **B.** MCF-7/TamR cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of peIF2α, GRP-78, pJNK2/1, CHOP, DR5 and GAPDH. Data represent at least three separately conducted experiments. **C.** MCF-7/TamR cells were transiently transfected with siRNA to CHOP, DR5 or JNK as well as a nonspecific negative control siRNA (labeled Control) using procedures described in Materials and Methods. Transfected cells were cultured for three days prior to collection of whole cell lysates for Western immunoblot analyses. siRNA to CHOP, DR5, and JNK blocked FASN chemical inhibitor C75-induced apoptosis as indicated by reduced PARP cleavage in comparison to siRNA control. Protein levels of pJNK, CHOP and DR5 were assessed to verify transfection efficiency. A, B, and C data represent three separately conducted experiments. **D**. MCF-7/parent, MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 at 2.5, 5 and 10 μM for 1 day. Apoptosis was determined by FACS analyses after annexin V/PI staining. Data are depicted as mean ± SD of three individual experiments.

Inhibition of FASN cooperates with α-TEA to induce apoptosis in TAMR cells

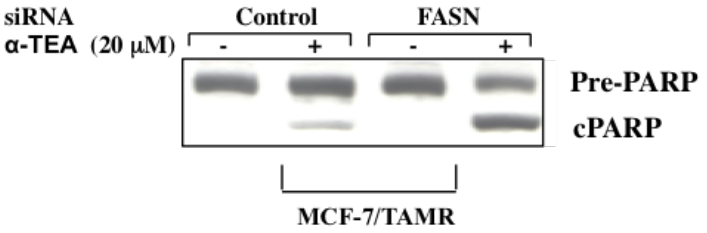
α-TEA suppresses FASN protein expression in both TAMR cell lines (Fig 47 A). Furthermore, the combination of FASN siRNA or C75 FASN inhibitor + α-TEA significantly increased levels of apoptosis detected by annexin V (Fig 47 B and D) and PARP cleavage (Fig 47 C and E) with CI at 0.26 and 0.51 for MCF-7/TamR and MCF-7/Her-2, respectively (Table 5). Furthermore, C-75 cooperates with α-TEA to induce increased levels of caspase-8 and caspase-9 cleavage (Fig 47 E), showing that combination of C75 + α-TEA induces caspase-8 and caspase-9 dependent apoptosis. These data suggest that the combination of α-TEA plus FASN inhibitor C75 will be effective in reducing TAM resistance in vivo.



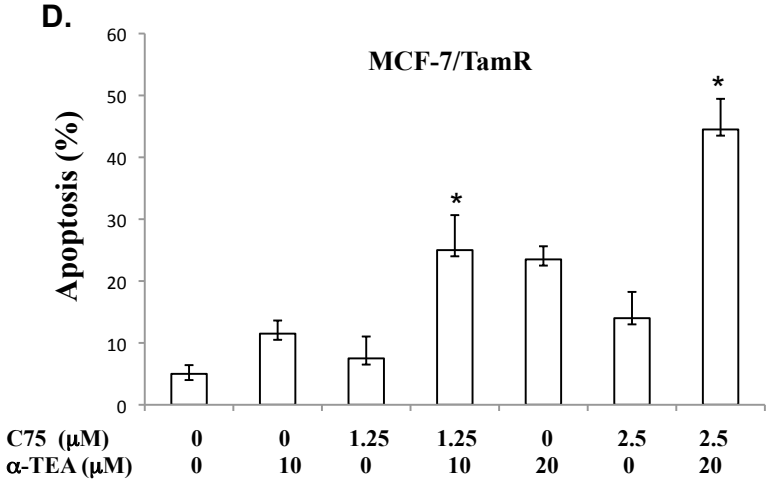
B.

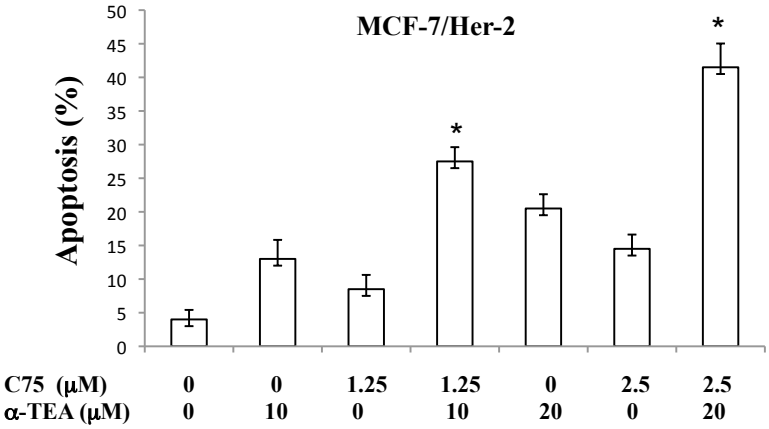

MCF-7/TamR
MCF-7/Her-2
Apoptosis (%)
60
50
40
30
20
10
0
*
*
FASN siRNA - + - + - + - +
α-TEA (20 μM) - - + + - - + +

C.


siRNA
α-TEA (20 μM)
Control
FASN
- + - +
Pre-PARP
cPARP
MCF-7/TAMR

D.


MCF-7/TamR
Apoptosis (%)
60
50
40
30
20
10
0
*
*
C75 (μM) 0 0 1.25 1.25 0 2.5 2.5
α-TEA (μM) 0 10 0 10 20 0 20


MCF-7/Her-2
Apoptosis (%)
50
45
40
35
30
25
20
15
10
5
0
*
*
C75 (μM) 0 0 1.25 1.25 0 2.5 2.5
α-TEA (μM) 0 10 0 10 20 0 20

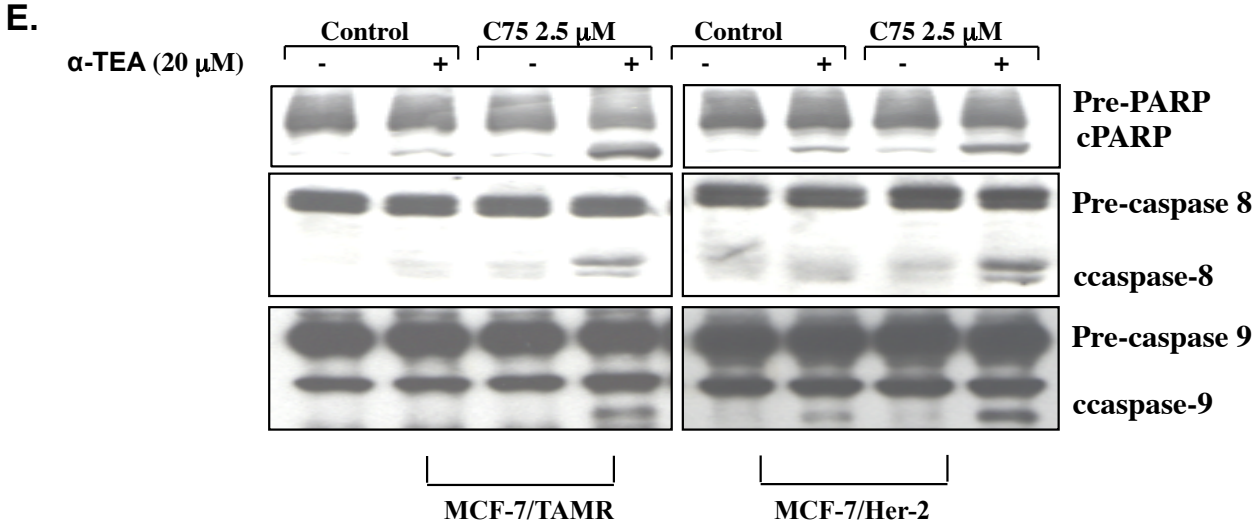


Figure 47: FASN inhibition cooperates with α-TEA to induce apoptosis. **A.** MCF-7/TamR and MCF-7/Her-2 cells were treated with 40 μM α-TEA for 9, 15 and 24 hours prior to collection of whole cell lysates for Western immunoblot analyses of FASN. **B.** MCF-7/TamR and MCF-7/Her-2 cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA and treated with 20 μM of α-TEA singly or in combination with FASN siRNA for 1 day prior to apoptosis analysis via annexin V/PI staining. **C.** MCF-7/TamR cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA and treated with 20 μM of α-TEA singly or in combination with FASN siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of PARP and FASN. **D.** MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 (1.25 or 2.5 μM), α-TEA (10 or 20 μM) and in combination as indicated for one day 1 day prior to apoptosis analysis via annexin V/PI staining. **E.** MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 (2.5 μM), α-TEA (20 μM) and in combination as indicated for one day for Western blot analyses of PARP, caspase-8 and caspase 9. Data in A, C and E are representative of three separately conducted experiments. B and D data are depicted as mean ± SD of three individual experiments. * = statistically different from control and individual treatments, $p<0.05$.

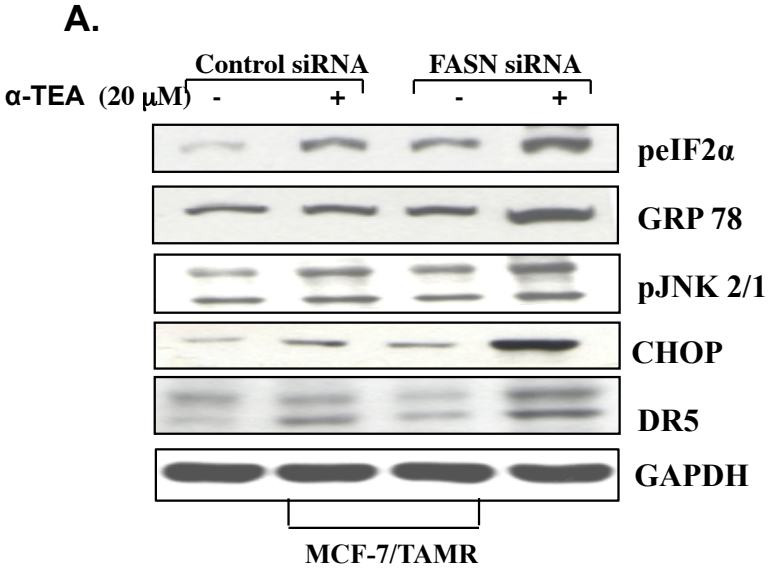
Table 5 Combination Index (CI) of apoptosis [a]

	α-TEA:C75 Ratio [b]	CI [c]			[d] Mean ± SD	
		ED50	ED75	ED90		
MCF-7/TamR	8:1	0.47	0.21	0.10	0.26 ± 0.19	Synergism [e]
MCF-7/Her-2	8:1	0.82	0.46	0.27	0.51 ± 0.27	Synergism [e]

[a]MCF-7/TamR and MCF-7/Her-2 breast cancer cells were treated with different concentrations of α-TEA and C75 alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining /FACS assay as described in Material and Methods.
[b]The ratio for the concentrations that were used in combination treatments were determined from the data in Fig1.
[c]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).
[d]The mean ± SD is calculated from the CI values of ED50, ED75 and ED90.
[e]CI values < 1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

FASN inhibition cooperates with α-TEA to induce ER stress mediated apoptosis

Combination of FASN siRNA or C75 inhibitor + α-TEA act cooperatively to induce increased levels of ER stress mediators peIF2α, GRP78, pJNK2/1, CHOP, and DR5 (Fig 48 A and B). siRNAs to CHOP or DR5 blocked α-TEA + FASN inhibitor C75 combination induced apoptosis as determined by reduced levels of cleaved PARP. Reduced levels of CHOP and DR5 showed efficacy of siRNAs. (Fig 48 C). These data suggest that the combination of α-TEA + FASN inhibitor/siRNA act cooperatively to induce ER stress mediated apoptosis.



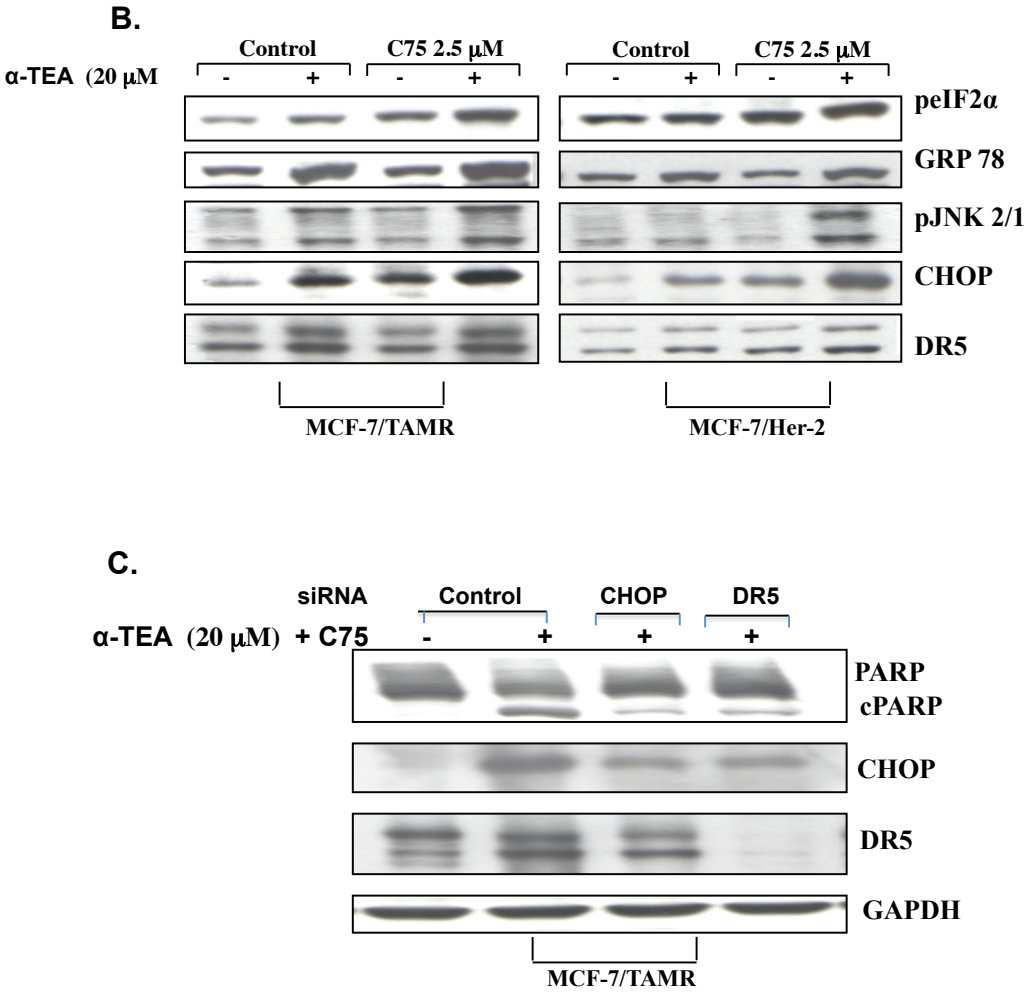
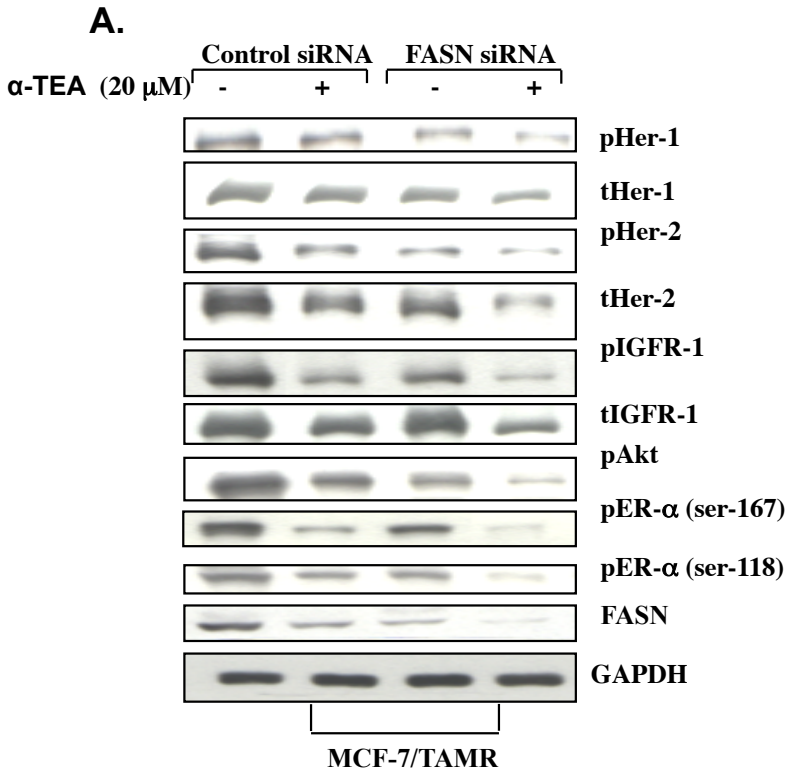


Figure 48: FASN inhibition cooperates with α-TEA to induce ER stress. **A.** Western immunoblot analyses using aliquots of cell lysates used in Figure 6C were performed to assess siRNA knockdown of FASN on ER stress mediators peIF2α, GRP-78, pJNK2/1, CHOP, and DR5. **B.** Western immunoblot analyses using aliquots of cell lysates used in Figure 6E were performed to assess FASN inhibitor C75 on ER stress mediators, namely peIF2α, GRP-78, pJNK, CHOP, and DR5. **C.** MCF-7/TamR cells were transiently transfected with siRNA to either CHOP or DR5 as well as nonspecific negative control siRNA (labeled Control) using procedures in Materials and Methods. Transfected cells were cultured for three days in the presence of α-TEA (20 μM) plus FASN inhibitor C75 prior to collection of whole cell lysates for Western immunoblot analyses. siRNA to both CHOP and DR5 blocked C75 + α-TEA induced apoptosis as indicated by PARP cleavage. Proteins level of CHOP and DR5 were assessed to verify transfection efficiency. Data are representative of three separately conducted experiments

FASN inhibition cooperates with α-TEA to suppress growth factor/survival factors

α-TEA in combination with siRNA knockdown of FASN or FASN inhibitor C75 suppress growth factor/survival factors pHer-1/total Her-1, pHer-2/total Her-2, pIGF1R/total IGF1R, pAkt and pER-α (Ser-118 and Ser-167) (Fig 49 A and B).



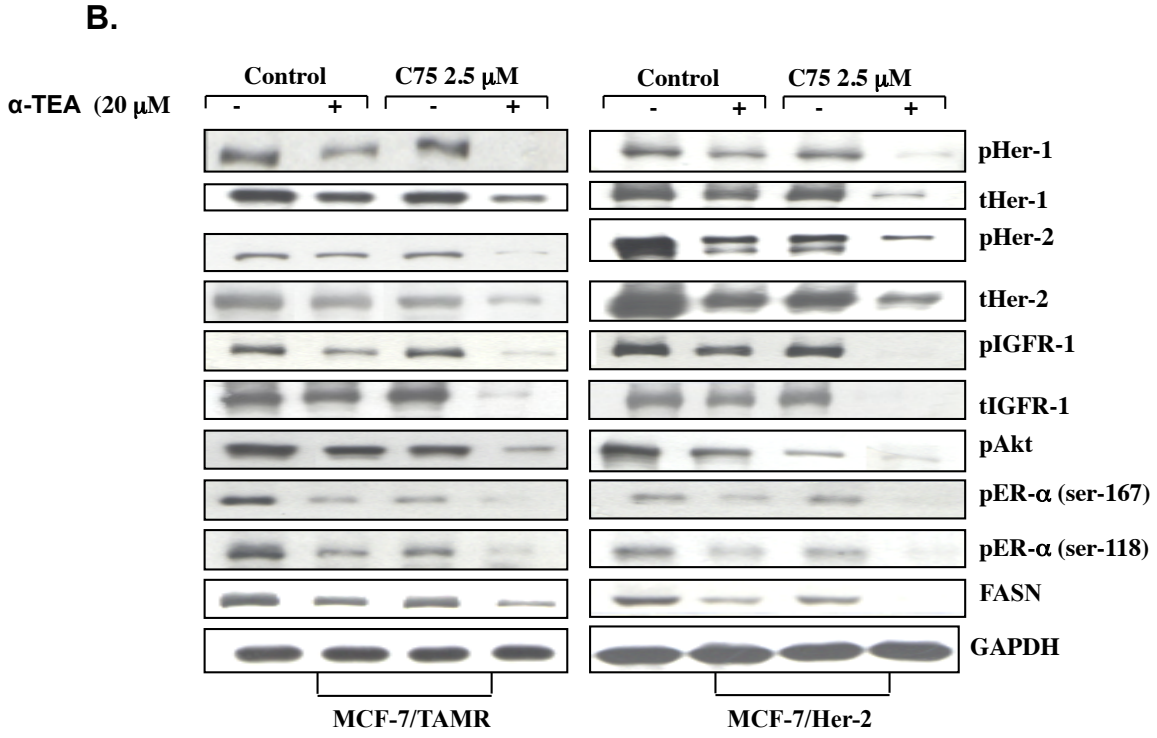


Figure 49: FASN inhibition cooperates with α-TEA to suppress pro-survival/anti-apoptotic factors. **A & B.** Western immunoblot analyses using aliquots of cell lysates used in Figure 6C (**A**) and aliquots of cell lysates used in Figure 6E (**B**) were performed to assess mediators of pro-survival loop, namely pHer-1/total Her-1, pHer-2/total Her-2, pIGFR/total IGFR, pAkt, pERα (ser-167), pERα (ser-118) as well as FASN expression levels. Data are representative of three separately conducted experiments.

6.4. Discussion

One of the many challenges for breast cancer therapy is to prevent and circumvent ET resistance. Accumulating data suggest that FASN is a novel target for breast cancer prevention and therapy [Lee et al., 2009; Campa et al., 2009]. However, there is a lack of knowledge on the role(s) that FASN plays in ET resistance, and if FASN can be a target to prevent and circumvent ET resistance. In this study, we demonstrated that FASN via cross-talk with growth factor mediators contributes to maintaining TAMR, and that suppression of FASN induces apoptosis in TAMR cells. Novel findings are: (i) FASN crosstalk with growth factor mediators contributes to TAM resistance, (ii) Inhibition of FASN induces TAMR cells to undergo apoptosis via activation of ER stress mediated JNK/CHOP/DR5 pro-apoptotic pathways, (iii) α-TEA functions as FASN inhibitor and cooperates with FASN siRNA or FASN inhibitor C75 to induce apoptosis mediators. Taken together, our data show that anticancer agents such as α-TEA that suppresses FASN in TAMR cells provides a treatment regimen for circumventing TAMR in human ER-positive TAMR breast cancers.

It is well established that TAMR is caused by over expression of receptor tyrosine kinase (RTK) proteins such as Her-1 and Her-2 and crosstalk among growth factor/survival mediators (membrane associated ER-α and Her-1 and Her-2 [Gee et al., 2005], Her-1/Her-2 and IGF1R [van der Veeken et al., 2009], and growth factor receptors and nuclear ER-α [Santen et al., 2009] are involved in

TAMR. Targeting specific components in these disordered, highly interactive, growth/survival pathways, such as Her-1, Her-2, or Akt using chemical inhibitors or blocking antibodies have been reported to circumvent TAM resistance [Marshall et al., 2006; Ghayad et al., 2010]. Our data show that FASN is highly expressed in TAMR cells in comparison with TAMS cells, is enhanced by TAM treatment, and that inhibition of FASN by treating the TAMR cells with α-TEA or knockdown of FASN using FASN siRNA and FASN inhibitor C75 suppresses Her-1, Her-2, IGF1R, pER-α and pAkt growth factors in TAMR cells. Interestingly, our data show that individual siRNA knockdown of Her-1, Her-2, IGF1R, pER-α or pAkt reduces FASN protein levels and induces apoptosis, suggesting critical crosstalk interactions between FASN and growth factor mediators in maintenance of TAMR. Reports have shown that FASN can be regulated by SREBP-1 via Akt and ERK [Swinnen et al., 2000]. FASN regulates Her-1 and Her-2 at both mRNA and phosphorylation levels [Vazquez-Martin et al., 2008]. Since Akt and ERK are regulated by Her-1 and Her-2, there is a positive amplification loop (Akt/FASN/Her-1/Her-2) in TAMR. It is well established that ER-α activity is regulated by Akt and ERK via phosphorylation of ER-α at ser-118 and ser-167, respectively, leading to increased ER-α transcriptional activity [Campbell et al., 2001; Murphy et al., 2004]. Studies show that IGF1R is one of the ER-α dependent genes and regulates Akt via PI3K [Umayahara et al., 1994], thereby identifying another positive amplification loop

(Akt/ER-α/IGF1R) in TAMR. In a schematic diagram (Fig 50), we propose that Akt acts as a bridge to link these two positive loops loops, leading to crosstalk among the growth factors and these two positive loops (Fig 50). This model describes the crosstalk identified in these studies among the growth factors, and demonstrates that FASN is a major contributor to TAMR. The evidence that inhibition of FASN using FASN siRNA or FASN inhibitor C75 in combination with TAM circumvents TAMR provides further support for a major role for FASN in TAMR. Inhibition of FASN not only suppressed TAMR survival mediators, but also cooperated with α-TEA to suppress TAMR survival signaling. These data also suggest that TAMR cells are "addicted" to highly interactive proliferation/survival mediators such that inhibition of a single proliferation/survival mediator leads to circumvention of TAMR and to cell death by apoptosis.

Beside FASN crosstalk with growth factor mediators, inhibition of FASN leads to cell death by apoptosis. We propose that FASN pro-apoptotic inducing property is important for circumvention of TAM resistance. FASN has been reported to induce apoptosis via ER stress [Little et al., 2007]. It has been established that lipid synthesis pathways are important for ER homeostasis and inhibition of phospholipid synthesis, especially that of phosphatidylcholine, induces ER stress-related pathways [Swinnen et al., 2003]. However, how ER stress pathways regulate FASN inhibition induced apoptosis is not clear. Here, we

show that JNK/CHOP/DR5 pro-apoptotic pathway is involved in FASN inhibition induced ER stress mediated apoptosis.

α-TEA, a pro-apoptotic inducer, exerts its anticancer functions via its dual actions; activation of pro-apoptotic pathways; Fas, ER stress mediated JNK/CHOP/DR5, p73/Noxa, as well as suppression of anti-apoptotic factors [Jia et al., 2008; Wang et al., 2008, Tiwary et al., 2010]. In this study, we found that α-TEA suppresses growth factor positive amplification loops, including FASN in TAMR cells. Our data show that α-TEA functions as a FASN inhibitor in TAMR cells as well as TAMS cell lines (data not shown). However, we do not know the initiating event(s) of α-TEA in the disruption of these positive amplification loops and crosstalk. Cholesterol-enriched lipid membrane rafts/microdomains are under investigation: α-TEA disrupts cholesterol lipid raft microdomains, platforms that harbor proliferation/survival mediators conducive for crosstalk between growth factor receptors and membrane ER (mER), [Marquez et al., 2006]. Support for disruption of cholesterol rich lipid microdomains to explain α-TEA circumvention of TAMR comes from data showing that α-TEA treatment of TAMR cells decreases cholesterol lipid raft marker DilC-16 and that exogenous cholesterol blocks α-TEA induced apoptosis [Chapter 5].

Taken together, our data show that FASN is a novel therapeutic target for TAMR breast cancers, and that α-TEA plus FASN chemical inhibitor C75 provides a combination treatment regimen for TAMR cancers.

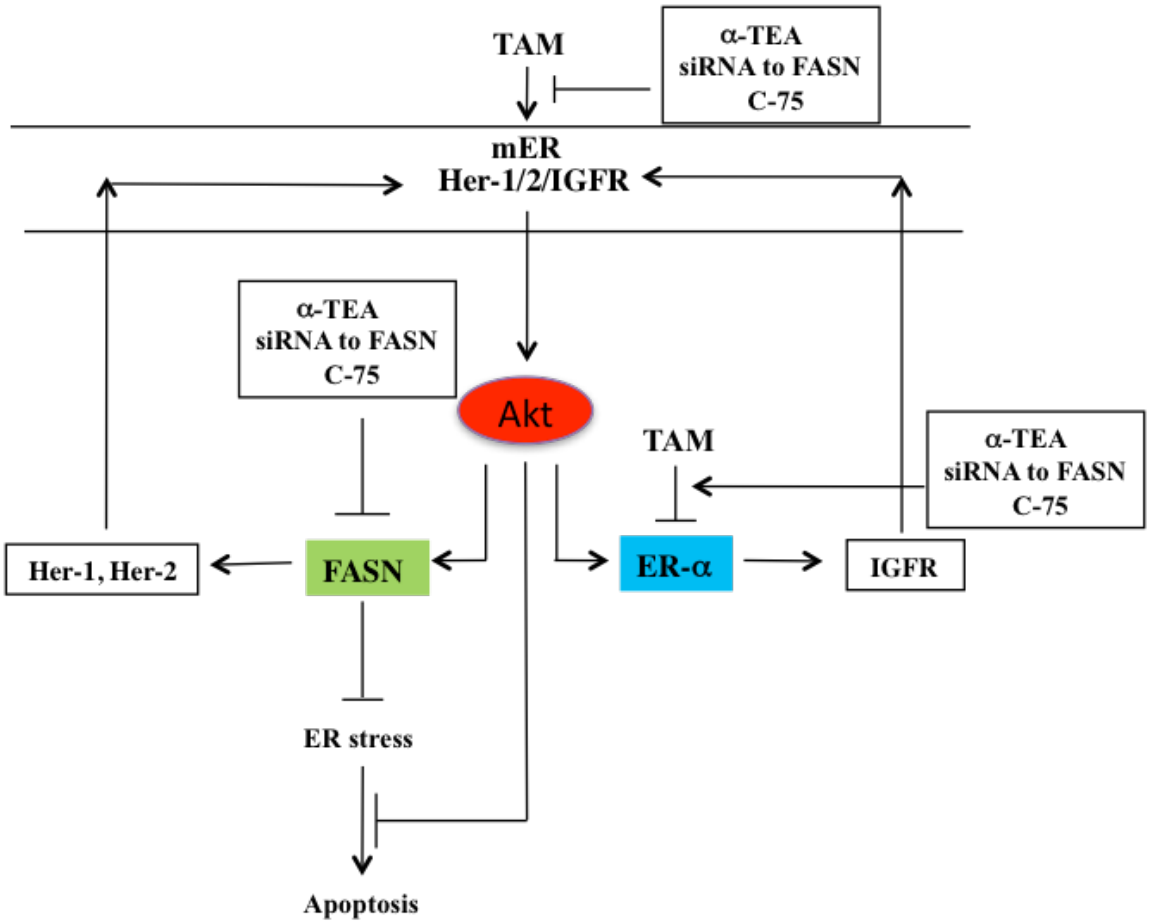


Figure 50: Proposed TAMR resistance signature; the involvement of FASN. Based on published data as well as date presented here, we propose that two positive loops: FASN/Her-1/Her-2/Akt/FASN and ER-α/IGF1R/AKT/ER-α and their crosstalk via Akt form an amplified survival signaling network to maintain TAMR. TAM stimulates TAMR survival network via membrane associated ER (mER) crosstalk with Her-1/Her-2. FASN not only plays an important role in this survival network, but also produces necessary lipid mediators to maintain endoplasmic reticulum (ER) homeostasis. Therefore, inhibition of FASN disrupts not only the amplified TAMR survival network, but also ER homeostasis to induce ER stress leading to ER stress mediated apoptosis. Akt, a pleiotropic pro-survival factor, blocks ER stress mediated apoptosis via different Akt downstream anti-apoptotic factors (not depicted). Thus, targeting FASN circumvents TAMR when combined with TAM by induction of apoptosis via activation of ER stress mediated apoptosis and suppression of survival network mediated anti-apoptotic factors. Since inhibition of FASN suppresses highly amplified Her-1/Her-2, TAM cannot stimulate TAMR survival network. Thus, its agonistic function is abrogated and its antagonistic function cooperates with FASN inhibition to suppress the survival network, which plays a critical role in circumventing TAMR.

Chapter 7
Conclusions and Future directions

7.1. Conclusions

Breast cancer is the most frequently diagnosed type of cancer in women, excluding non-melanoma skin cancers. An estimated 207,090 new cases of invasive breast cancer and 54,010 new cases of in situ breast cancer are expected among women in US in 2010. Moreover, breast cancer is the second leading cause of mortality among women in North America. An estimated 40,230 breast cancer deaths are projected to occur in the US in 2010 [American Cancer Society, Surveillance and Health Policy Research 2010]. On a global scale, it is estimated that breast cancer will occur in more than five million women worldwide over the next decade.

Clearly, there is an urgent need for new strategies for prevention of primary cancers, treatment, and prevention of cancer recurrence in survivors. As we better understand the complexities of cancers, i.e., amplified pro-survival mediators, suppressed pro-death mediators, and the heterogeneity of cancer cells within tumors, it becomes increasingly clear that although single treatments or agents are effective initially, tumor relapse occurs in many patients. Current wisdom is that combinations of treatments, each contributing in an additive or synergistic manner are more effective methods for treating heterogeneous tumors. Goals of these studies were to further investigate the anticancer actions of α-TEA in order to

obtain a better understanding of the survival and death signaling mediators impacted, and to explore α-TEA's contributions to cancer cell death by apoptosis when in combination with chemotherapeutic agents, endocrine therapy, rapamycin, PI3K inhibitor (wortmannin), MEK inhibitor (U01260), FASN inhibitor (C75) and lipid raft disruptor (MβCD).

α-TEA is structurally similar to its parent compound, natural vitamin E (RRR-α-tocopherol), differing by having a non-hydrolysable (i.e. stable) ether-linked acetic acid moiety at carbon 6 of the chroman head, thereby altering the C6 hydroxyl moiety responsible for anti-oxidant potential. α-TEA has been shown to be effective at reducing tumor burden and metastasis in xenograft models of transplanted human breast cancer cells, human prostate cancer cells or cisplatin-resistant human ovarian cancer cells as well as in syngeneic mouse mammary tumor models [66-70]. Importantly, α-TEA does not induce apoptosis in normal human prostate epithelial cells (PrEC) or normal human mammary epithelial cells (HMEC), making it an ideal chemotherapeutic agent [65]. Several recent reports have shown α-TEA to be an effective inhibitor of tumor and metastatic growth *in vivo* [40-44].

Chapter 2 provides data on the underlying mechanisms of how α-TEA enhances DR5 death signaling mediators via induction of endoplasmic reticulum (ER) stress, as determined by increased expression of CCAAT/enhancer binding protein homologous protein (CHOP) as well as by enhanced expression or

activation of specific markers of ER stress such as glucose regulated protein (GRP78), phosphorylated alpha subunit of eukaryotic initiation factor 2 (peIF-2α), and spliced XBP-1 mRNA. These data, for the first time, demonstrate that: i) α-TEA activates a DR5/caspase-8, ER stress mediated JNK/CHOP/DR5 positive amplification loop, and ii) down-regulates anti-apoptotic mediator c-FLIP (L) protein levels which is mediated by a JNK/CHOP/DR5 amplification loop via a JNK dependent Itch E3 ligase ubiquitination that further serves to enhance the JNK/CHOP/DR5 amplification loop by preventing c-FLIP's inhibition of caspase-8, and (iii) α-TEA down-regulation of Bcl-2 is mediated by the ER stress dependent JNK/CHOP/DR5 signaling. Taken together our data show that ER stress via JNK/CHOP/DR5/ caspase-8 signaling plays an important role in α-TEA induced apoptosis by enhancing DR5/caspase-8 pro-apoptotic signaling and suppressing anti-apoptotic factors c-FLIP and Bcl-2.

Chapter 3 shows the ability of α-TEA alone and in combination with inhibitors of Akt (wortmannin), MEK inhibitor (U01260) or mTOR (rapamycin) to inhibit pro-survival mediators and induce apoptosis in MCF-7 and HCC-1954 breast cancer cell lines. Data show that α-TEA suppressed constitutively active basal levels of pAKT, pERK, pmTOR, and their down-stream targets, as well as induced both cell types to undergo apoptosis. PI3K inhibitor Wortmannin suppressed pAKT, pERK and pmTOR and downstream targets, indicating PI3K to be a common upstream mediator. α-TEA induced increased levels of pIRS-1

(Ser-307), a phosphorylation site correlated with IRS-1 inactivation, and decreased levels of total IRS-1. JNK blocked α-TEA's impact on pIRS-1 and total IRS-1 and impeded α-TEA's ability to downregulate the phosphorylated status of AKT, ERK and mTOR. Combinations of α-TEA + MEK or mTOR inhibitors acted cooperatively to induce apoptosis and reduce basal levels of pERK and pmTOR, respectively. Importantly, inhibitors of MEK or mTOR resulted in increased levels of pro-survival pAKT and IRS-1 and α-TEA blocked both. In summary, α-TEA's ability to target PI3K mediated pro-survival factors via JNK mediated inhibition of IRS-1 not only plays a role in enhancing α-TEA induced apoptosis by inhibition of AKT, ERK and mTOR and their downstream mediators, but also suggests a novel means for preventing pro-cancer impact of AKT activation induced by ERK and mTOR inhibitors.

Chapter 4 data show the anticancer effects of chemotherapeutic drugs and α-TEA separately and in combination on p53 mutant, triple negative breast cancer (TNBC) cell lines, MDA-MB-231 and BT-20. Successful treatment of p53 mutant, TNBCs remains a challenge. Doxorubicin (DOXO) and cisplatin (CDDP) are standard-of-care treatments for TNBC, but eventually fail due to acquired drug resistance and toxicity. Therefore, new treatments for overcoming drug resistance and toxicity in p53 mutant, TNBC are a high priority. Here we report on the reconstitution of the p53 tumor suppressor pathway in a p53-independent manner via p53 homologue p73 with combination treatments of α-TEA plus

DOXO or CDDP. Combination treatments of α-TEA plus DOXO or CDDP act cooperatively to induce apoptosis, caspase-8 and -9 cleavage, elevated p73, phospho-c-Ab1 and phospho-JNK protein expression, and elevated expression of p53 downstream mediators; namely, death receptor-5 (DR5), CD95/APO-1 (Fas), Bax and Noxa, as well as Yap nuclear translocation, plus reduced expression of anti-apoptotic mediator Bcl-2. Based on siRNA knockdown of p73, we conclude that p73 plays a critical role in combination treatment enhanced apoptosis of TNBC p53 mutant cancers, via p53 independent activation of p53 mediated pro- and down regulation of anti-apoptotic mediators, and that c-Abl, JNK and Yap are upstream mediators of p73 in combination treatment responses.

Chapter 5 focuses on how α-TEA circumvents TAM-resistance (TAMR) in acquired and de novo TAMR human breast cancer cells, the most common cause of treatment failure for women with estrogen receptor-positive (ER+) breast cancers. TAMR molecular signatures of MCF-7/TAMR breast cancer cells, in comparison to MCF-7 TAM sensitive (MCF-7/TAMS) breast cancer cells included: highly expressed cholesterol-rich cell membrane lipid domains and highly amplified signaling mediators for growth factor receptors (total and phosphorylated forms of Her-1, Her-2 and IGFR-1), and increased expression of activated (phosphorylated forms) of proliferation/survival signal transduction molecules (pAkt, pmTOR, and pERK 1/2). TAM treatment alone enhanced pro-survival signaling mediators; whereas, α-TEA treatment alone reduced

cholesterol rich microdomains, reduced pro-survival mediators and induced apoptosis. α-TEA + TAM combination treatment of TAMR cells cooperatively acted to reduce proliferation/pro-survival mediators and induced the cells to undergo death receptor (DR5) mediated mitochondrial dependent apoptosis via activation of endoplasmic reticulum stress amplification loop (pJNK/CHOP/DR5). Further support for these data come from studies showing that lipid raft disruptor, methyl-β-cyclodextrin (MβCD) synergizes with both TAM and α-TEA to induce apoptosis via inhibiting proliferation/pro-survival mediators, and treatment of TAMR cells with exogenous cholesterol blocks α-TEA plus TAM induced apoptosis. Thus, we conclude that α-TEA + TAM circumvents TAM resistance by inducing apoptosis via activation of endoplasmic reticulum stress/DR5 death receptor amplification loop mediated pro-apoptotic pathway and suppression of cholesterol rich membrane microdomains, followed by down-regulation of proliferation/survival mediators as well as anti-apoptotic mediators.

Chapter 6 data show that fatty acid synthase (FASN) is involved in crosstalk among growth factors in TAM resistant cell lines, and that inhibition of FASN circumvents TAM resistance. Both TAM resistant cell lines express higher levels of FASN protein in comparison with their TAM sensitive counterparts. TAM induces increased levels of FASN in TAM resistant cells and inhibits FASN in TAM sensitive cells. Inhibition of FASN in the two TAM resistant cell lines by

siRNA knockdown or treatment with FASN inhibitor (C75), followed by TAM treatment show cooperative actions of such treatments in the circumvention of TAM resistance. α-TEA alone suppresses FASN protein expression, and cooperates with knockdown of FASN (siRNA or chemical inhibitor) to induce apoptosis in both resistant cell lines. α-TEA inhibition of FASN in TAM resistant cell lines suppresses growth factor mediators Her-1, Her-2, IGFR-1, pAkt, and pER-α. siRNA knockdown of Her-1, Her-2, IGFR-1, Akt, or ER-α blocks FASN protein expression, suggesting crosstalk and a positive amplification loop between FASN and other pro-survival factors in TAM resistant cell lines. Furthermore, combinations of α-TEA plus TAM in TAM resistant cell lines where FASN has been knocked down using siRNA or chemical inhibitor produce enhanced activation of ER stress mediated JNK/CHOP/DR5 pro-apoptotic mediators and suppression the anti-apoptotic mediators. Taken together, these data showed that targeting FASN has potential as a novel strategy to circumvent TAM resistance when combined with TAM, and that the combination of α-TEA (which functions as a FASN inhibitor) plus FASN inhibitor provide a novel strategy for treatment of TAM resistant breast cancers.

7.2. Future Directions

These studies provide important insights into the complex signaling mediators that regulate cell survival, proliferation, resistance and apoptosis in human breast cancer cells, and provide strong data supporting α-TEA combination regimens for breast cancer treatment. There are several important findings from this research study that warrant further investigations.

(i) Validate the efficacy of the combination of α-TEA + TAM in preventing acquired TAM resistance, and combination treatment effects on de novo and acquired TAM resistant tumors using *in vivo* xenograft models.

(ii) Determine the anti-cancer efficacy of α-TEA + TAM on targeting cancer stem cells (CSC) also referred to as tumor initiating cells (TIC) and metastases *in vitro* and *in vivo*. Our preliminary data show that TAMR cells are enriched in CSC (using mammospheres, CD44+/CD24- staining and ALDH assay), and that α-TEA eliminates CSCs as determined by inhibition of serial mammosphere formation.

(iii) Determine the anti-cancer actions of natural occurring FASN inhibitor (Epigallocatechin Gallate, ECGC, isolated from green tea) and natural occurring cholesterol lipid raft disruptor (Docosahexaenoic acid, DHA isolated from omega-3 fatty acids) separately and in combination on circumventing TAMR when combined with TAM *in vitro* and *in vivo*. Based on our data showing that inhibition of FASN and disruption of cholesterol-rich lipid raft microdomains can

circumvent TAMR, we hypothesize that natural occurring FASN inhibitor ECGC plus cholesterol lipid raft disruptor DHA plus tamoxifen will circumvent TAMR.

(iv) Validate the anti-cancer efficacy of the combination of α-TEA + doxorubicin or cisplatin drugs as treatments for triple negative human breast cancer using *in vivo* models.

(v) Determine the ability of the combination of α-TEA + doxorubicin to eliminate both CSC and non-CSC (bulk) cancer cells in triple negative breast cancers in vitro and in vivo. Rationale comes from studies showing that chemotherapeutic agents induce non-stem bulk cancer cells to undergo apoptosis but enrich CSCs.

(vi) Determine α-TEA initiating event leading to induction of apoptosis in human breast cancer cells.

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Appendix #1. Cooperative interactions between FASN inhibitors and tamoxifen (TAM) to circumvent tamoxifen resistance (TAMR)

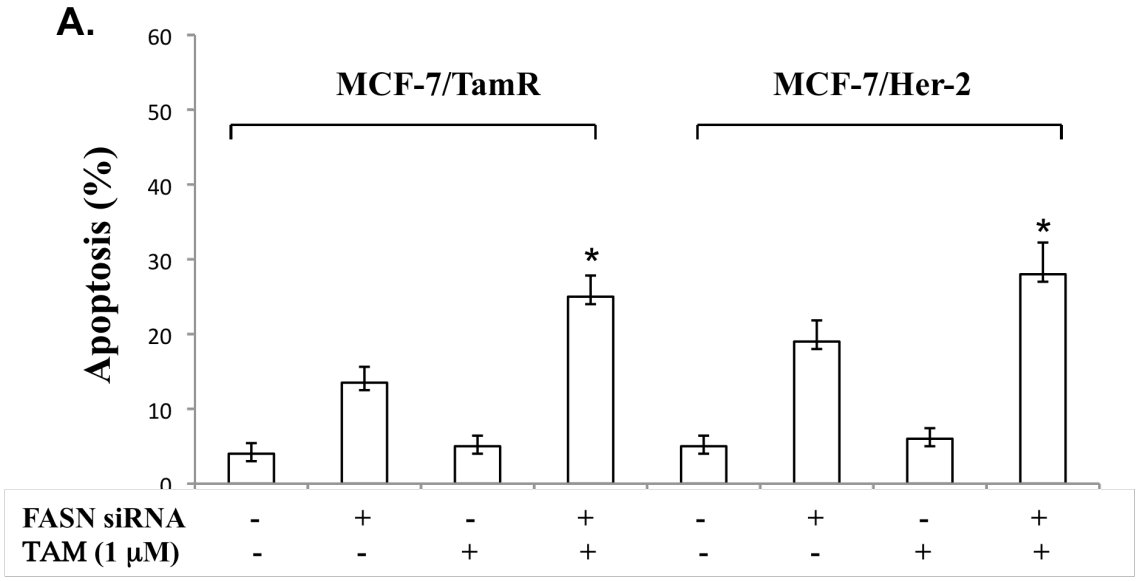
Appendix #1 contains data generated during the conduct of the studies described in Chapter 6. The introduction, human breast cancer cell lines, and procedures used for generating the data presented here are provided in Chapter 6. Focus of these data are on the cooperative interactions of tamoxifen (TAM) plus inhibitiors of fatty acid synthase (FASN) on circumventing TAMR in human TAMR breast cancer cell lines. The data (Results) are presented in 3 Figures and 1 Table. Data for each Figure and Table are analyzed. A brief discussion concludes Appendix #1.

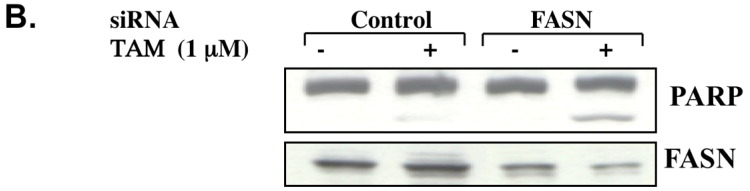
Results

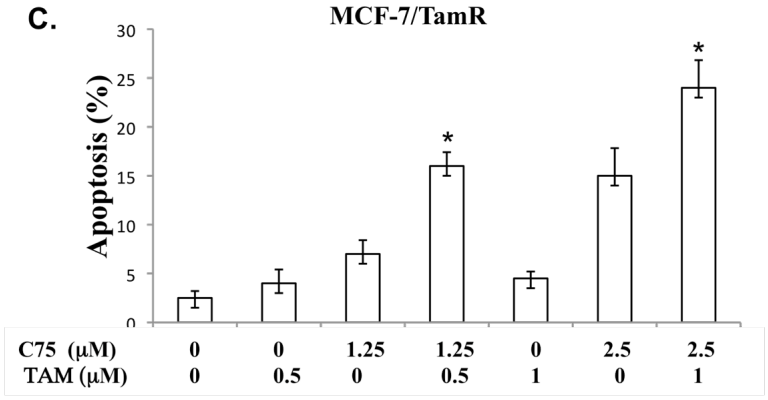
Inhibition of FASN using FASN C75 inhibitor or siRNA to FASN plus TAM act cooperatively to induce apoptosis

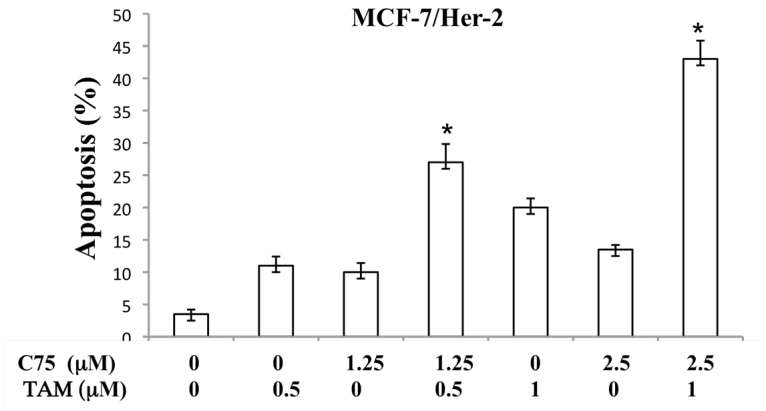
Combination of FASN siRNA or inhibitor C75 + TAM induced increased levels of apoptosis as detected by annexin V (Fig 1A and C) and PARP cleavage (Fig 1B and D) with CI (combination index) at 0.19 and 0.08 for MCF-7/TamR and MCF-7/Her-2 cells, respectively (Table 1), suggesting a synergistic effect on induction of apoptosis.

Furthermore, FASN inhibitor C-75 cooperates with TAM to induce increased levels of caspase-8 and caspase-9 cleavage (Fig 1D), showing that combination of C75 + TAM induces caspase-8 and caspase-9 dependent apoptosis.




B.
siRNA
TAM (1 μM)
Control
FASN
-
+
-
+
PARP
FASN


C.
MCF-7/TamR
Apoptosis (%)
30
25
20
15
10
5
0
*
*
C75 (μM) 0 0 1.25 1.25 0 2.5 2.5
TAM (μM) 0 0.5 0 0.5 1 0 1


MCF-7/Her-2
Apoptosis (%)
50
45
40
35
30
25
20
15
10
5
0
*
*
C75 (μM) 0 0 1.25 1.25 0 2.5 2.5
TAM (μM) 0 0.5 0 0.5 1 0 1

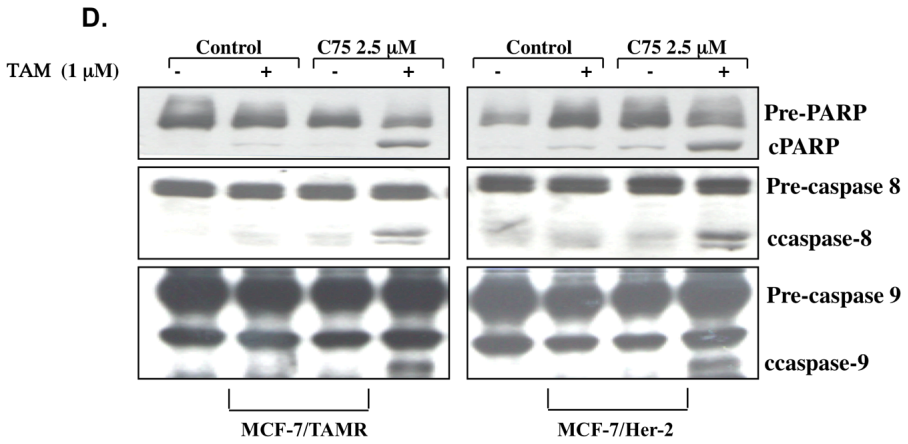


Figure 1: Inhibition of FASN induces apoptosis singly and in combination with TAM in TAMR cells. **A.** MCF-7/TamR and MCF-7/Her-2 cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA and treated with 1 μM of TAM singly or in combination with FASN siRNA for 1 day prior to apoptosis analysis via annexin V/PI staining. **B**. MCF-7/TamR cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA and treated with 1 μM of TAM singly or in combination with FASN siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of PARP and FASN. **C.** MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 (1.25 or 2.5 μM), TAM (0.5 or 1 μM) and in combination as indicated for one day 1 day prior to apoptosis analysis via annexin V/PI staining. **D.** MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 (2.5 μM), TAM (1 μM) and in combination as indicated for one day for Western blot analyses of PARP, caspase-8 and caspase 9. Data in A and C are depicted as mean ± S.D. of three independent experiments. * = Statistically different from control and individual treatments, $p<0.05$. B and D data are representative of three independent experiments.

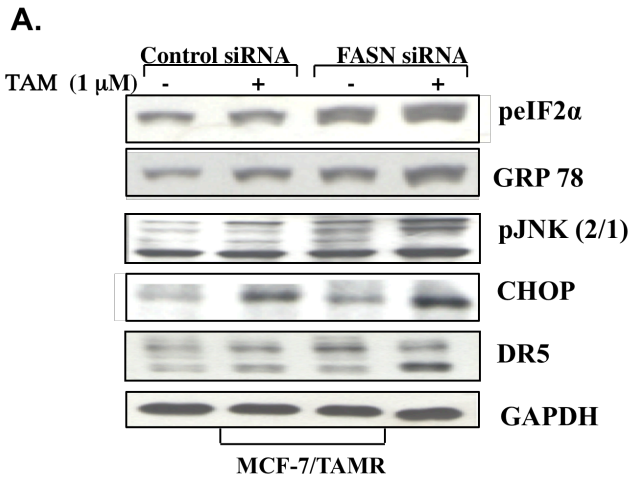
Table 1 Combination Index (CI) of apoptosis [a]

	C75:TAM Ratio [b]	CI [c]			Mean ± SD [d]	
		ED50	ED75	ED90		
MCF-7/TamR	2.5:1	0.23	0.19	0.16	0.19 + 0.04	Synergism [e]
MCF-7/Her-2	2.5:1	0.14	0.07	0.04	0.08 + 0.05	Synergism [e]

[a]MCF-7/TamR and MCF-7/Her-2 breast cancer cells were treated with different concentrations of TAM and C75 alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining /FACS assay as described in Material and Methods.
[b]The ratio for the concentrations that were used in combination treatments were determined from the data in Fig1.
[c]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).
[d]The mean ± SD is calculated from the CI values of ED50, ED75 and ED90.
[e]CI values < 1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

Combination of C75 or siRNA to FASN plus TAM act cooperatively to induce ER stress mediated pro-apoptotic pathway

Combination of FASN siRNA (Fig 2A) or C75 FASN chemical inhibitor (Fig 2B) cooperates with TAM to induce ER stress detected by ER stress markers peIF-2α and GRP78 as well as upregulate pJNK, CHOP and DR5 protein expression. siRNA knockdown of CHOP blocked C-75 + TAM combination induced apoptosis, as well as increase in CHOP protein expression (Fig 2C).



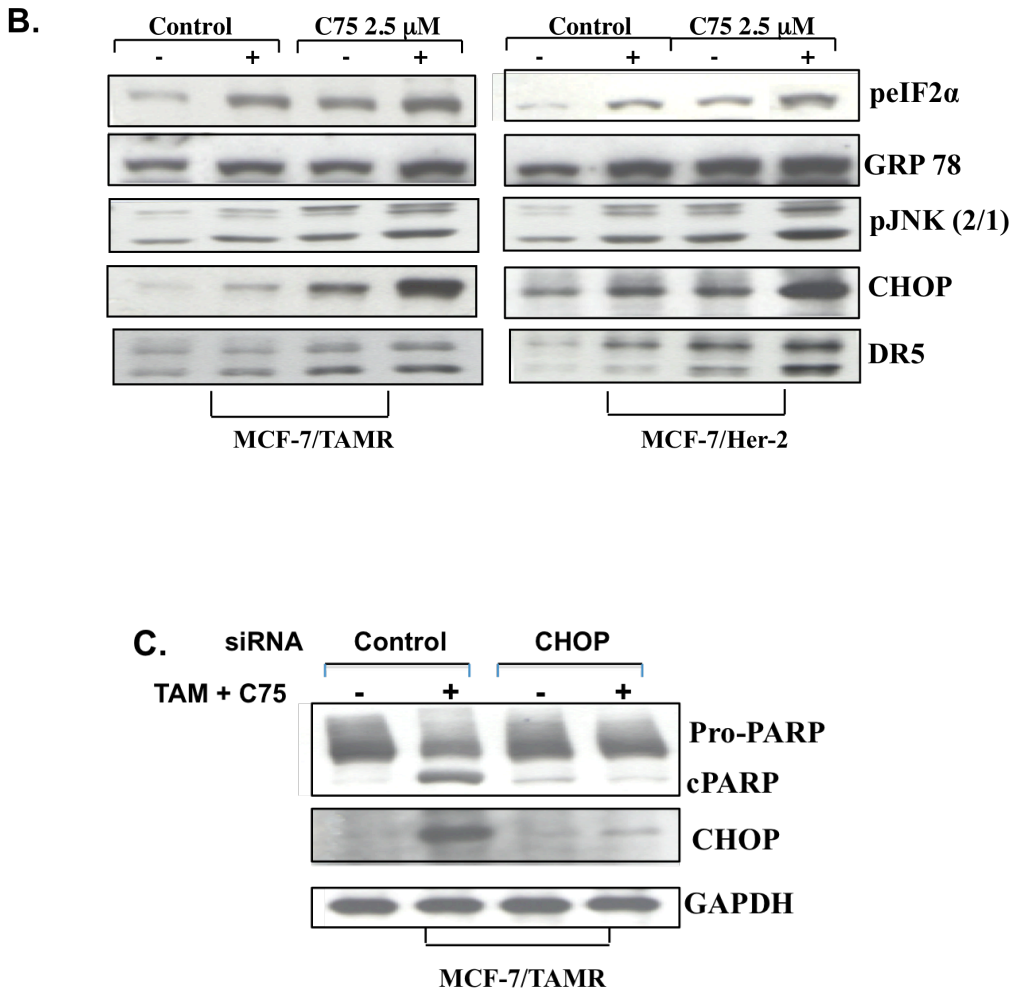
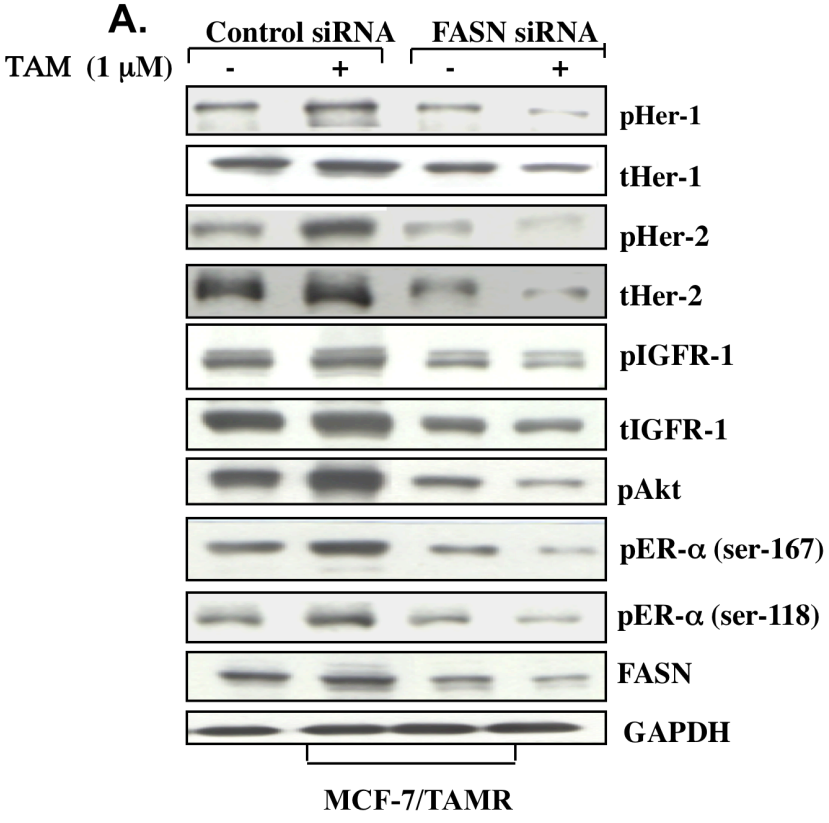


Figure 2. Combination of C75 or siRNA to FASN plus TAM act cooperatively to induce ER stress mediated pro-apoptotic pathway. **A.** Western immunoblot analyses using aliquots of cell lysates from Figure 1B were performed to assess ER stress mediators: peIF2α, GRP 78, pJNK2/1, CHOP, and DR5. GAPDH served as lane controls. **B.** Western immunoblot analyses using aliquots of cell lysates from Figure 2D were performed to assess ER stress mediators: peIF2α, GRP 78, pJNK2/1, CHOP and DR5. GAPDH served as lane controls. **C.** MCF-7/TamR cells were transiently transfected with siRNA to CHOP as well as a nonspecific negative control siRNA (labeled Control) using procedures described in Materials and Methods. Transfected cells were cultured for three days prior to collection of whole cell lysates for Western immunoblot analyses. siRNA to CHOP blocked C75 + TAM induced apoptosis as indicated by PARP cleavage. Proteins levels of CHOP were assessed to verify transfection efficiency. Data are representative of three independent experiments.

FASN inhibition cooperates with TAM to suppress pro-survival/anti-apoptotic factors

Combination of FASN siRNA or FASN chemical inhibitor (C75) + TAM not only prevents TAM induced increases in growth factor/survival factors, but also cooperatively acts to suppress these pro-survival mediators (Fig 3 A and B). These data demonstrate that inhibition of FASN converts TAM agonistic action to antagonistic action in regulating growth factor mediators, indicating that blockage of FASN sensitizes TAM resistance cells to TAM.



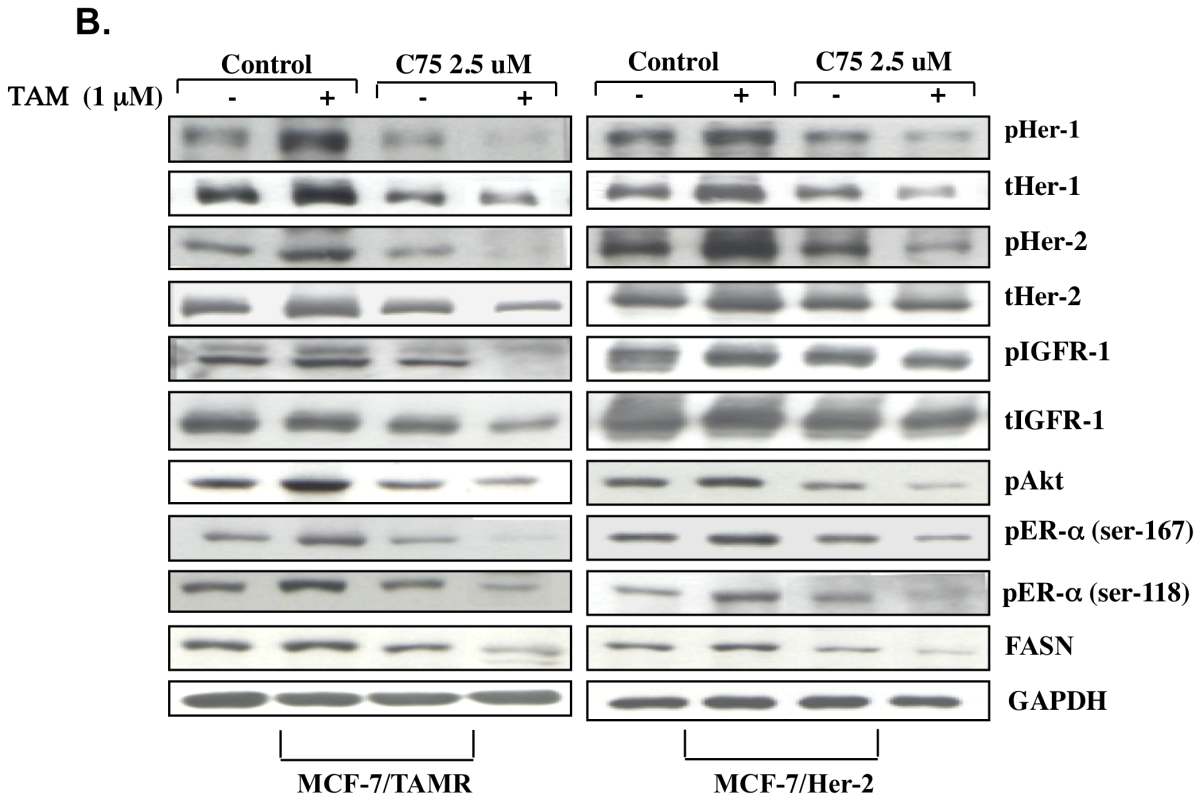


Figure 3: Inhibition of FASN cooperates with TAM to suppress pro-survival amplification loop. **A.** MCF-7/TamR cells were transiently transfected with siRNA to either FASN or a nonspecific negative control siRNA and treated with 1 μM of TAM singly or in combination with FASN siRNA for 1 day prior to collection of whole cell lysates for Western immunoblot analyses of pHer-1/Her-1, pHer-2/Her-2, pIGF1R/IGF1R, pAkt, pERα (ser-118 and ser-167), FASN and GAPDH. **B**. MCF-7/TamR and MCF-7/Her-2 cells were treated with C75 (2.5 μM), TAM (1 μM) and in combination as indicated for one day for Western blot analyses of pro-survival mediators mentioned above. Data are representative of three independent experiments.

Discussion

Endocrine therapy (ET) resistance remains as one of the major obstacles for breast cancer therapy. Recent reports have supported FASN as a novel therapeutic target for breast cancer prevention and therapy. However, role of FASN in ET resistance remains elusive. Therefore, we explored the possibility of FASN as a target to circumvent ET resistance. In this study, we demonstrated that FASN via cross-talk with growth factor mediators contributes to maintaining TAMR, and that suppression of FASN circumvents TAMR when combined with TAM. Data in Chapter 6 show that TAMR cells have elevated levels of FASN in comparison with TAMS cells and that FASN is further enhanced by TAM treatment.

Novel findings in this study are: (i) inhibition of FASN cooperates with TAM to induce apoptosis (ii) Combination of TAM + FASN inhibitors (siRNA/C75) induces ER stress mediated pro-apoptotic pathway, and (iii) Inhibition of FASN cooperates with TAM to suppress TAMR pro-survival signature pathway-pHer-1/Her-1, pHer-2/Her-2, pIGF1R/IGF1R, pERα, pAkt and FASN. Taken together, our data show that anticancer agents that suppress FASN in TAMR cells provides a treatment regimen for circumventing TAMR in human ER-positive TAMR breast cancers.

The evidence that inhibition of FASN using FASN siRNA or FASN inhibitor C75 in combination with TAM circumvents TAMR provides further support for a

major role for FASN in TAMR. Inhibition of FASN not only suppressed TAMR survival mediators, but also cooperated with TAM to suppress TAMR survival signaling, showing that suppression of FASN converts TAM from an agonist to an antagonist, thereby circumventing TAMR. These data also suggest that TAMR cells are "addicted" to highly interactive survival pathways such that inhibition of a single proliferation/survival mediator leads to circumvention of TAMR, and to cell death by apoptosis. Taken together, our data show that FASN is a novel therapeutic target for circumventing TAMR when combined with TAM, and that FASN chemical inhibitor C75 or FASN inhibitor plus TAM provide treatment regimens for TAMR cancers.

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