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Role of Autophagy in Mediating the Anticancer Effects of Tocotrienols

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Abstract

γ -Tocotrienol, a natural isoform of vitamin E, is a potent anticancer agent. Autophagy is a highly regulated process by which debris is eliminated from a cell, but can also play a role in cellular survival or death. The role of autophagy in mediating the anticancer effects of γ -tocotrienol is not clearly understood. This chapter reviews the mechanism(s) involved in γ -tocotrienol-induced autophagy in breast cancer cells. Treatment with γ -tocotrienol increased conversion of microtubule-associated protein, 1A/1B-light chain 3, from its cytosolic form (LC3B-I) to its lipidated form (LC3B-II), and the accumulation of autophagy-related proteins Beclin-1 (Atg6) and Atg5-Atg12. Additional studies confirmed that transfection with Beclin-1 siRNA or pretreated with 3-methyladenine (3-MA), an inhibitor of autophagy, blocked these effects. γ -Tocotrienol treatment also induced a time-responsive increase in autolysosome markers LAMP-1 and cathepsin-D, and pretreatment with bafilomycin A1 (Baf1), an inhibitor of late phase autophagy, blocked these effects and caused a significant reduction in γ -tocotrienol-induced cytotoxicity. γ -Tocotrienol also induced a decrease in ERK, an increase in p-38 and JNK activation, and endoplasmic reticulum (ER) stress apoptotic markers including phospho-PERK, phospho-elf2 α , Bip, IRE1 α , ATF-4, CHOP, and TRB3. In summary, γ -tocotrienol-induced autophagy is intimately involved in promoting ER-stress-mediated apoptosis in human breast cancer cells.

Keywords: γ -tocotrienol, autophagy, breast cancer, endoplasmic reticulum stress, oridonin

1. Introduction to autophagy

Autophagy (Greek for “self-eating”) is hallmarked by the formation of double-membrane-bound organelles known as autophagosomes and is a lysosome-dependent pathway for the degradation

of damaged cytoplasmic organelles and proteins [1]. There are multiple subtypes of autophagy that are classified according to mechanism and function [2, 3]. The present review will focus on macroautophagy, which is characterized by the engulfment and degradation of cytoplasmic materials in bulk in a selective/nonselective manner. Autophagy is the only mechanism that is involved in the degradation of large structures such as organelles and protein aggregates. In the absence of stress, autophagy serves a housekeeping function [2, 3]. However, during starvation autophagy provides material that can be used as a source of nutrition to promote survival. Autophagy can be induced by a broad range of other stressors to aid in the degradation of protein aggregates, oxidized lipids, damaged organelles, and even intracellular pathogens [2, 3]. Defects in autophagy are linked to liver disease, neurodegeneration, Crohn's disease, aging, cancer, and metabolic syndrome [4].

Autophagy is a self-catabolic cellular mechanism in which damaged cellular organelles in the cytoplasmic compartment are sequestered into the double-membrane vesicles known as autophagosomes that further fuse with lysosomes to form autophagolysosomes. Excessive formation of autophagic vesicles interferes with normal membrane functioning and can lead to autophagy-associated programmed cell death [3–5]. Autophagy is regulated by class I and class III phosphatidylinositol 3-kinase (PI3K)-signaling pathways. Activation of class I PI3K leads to the phosphorylation of plasma membrane lipids that play a role in the recruitment and activation of Akt, a downstream negative regulator of autophagy. The tumor suppressor, phosphatase and tensin homolog (PTEN), dephosphorylates lipids in the plasma membrane and thereby acts to prevent the activation of Akt [3–5]. Activated Akt inhibits the tuberous sclerosis complex 1 (TSC1) and TSC2 proteins that act as positive regulators of autophagy by repressing the activity of the small G protein Rheb, which modulates mammalian target of rapamycin (mTOR) [3–5]. mTOR functions to inhibit autophagy [3–5]. Although the exact mechanism by which mTOR inhibits autophagy is not completely understood, it appears to be involved in suppressing Atg autophagy-related genes [6].

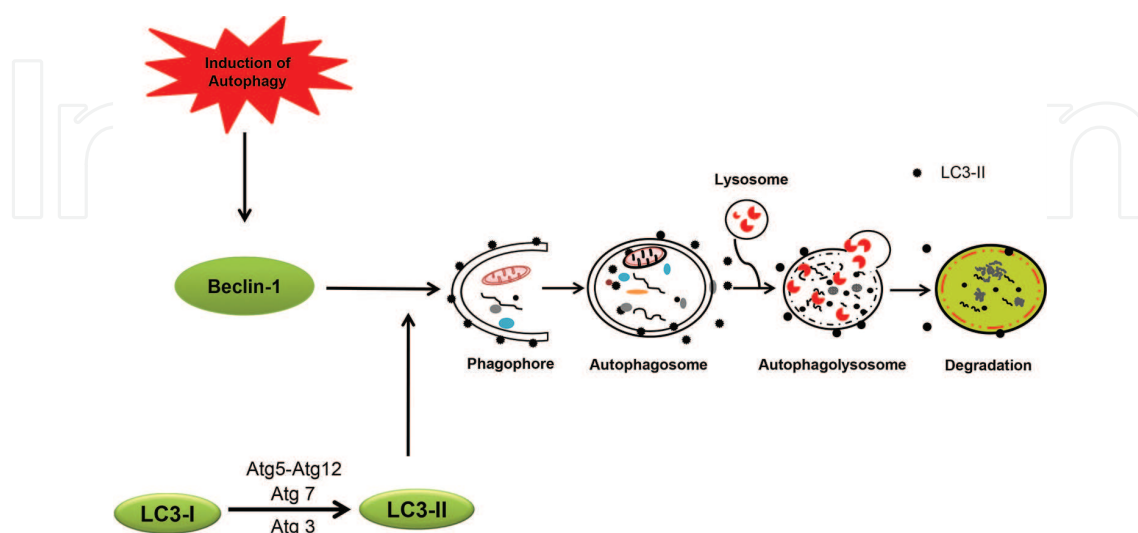


Figure 1. Schematic representation of autophagy.

The execution and regulation of the autophagy pathway is governed by Atg [6]. The initial nucleation and assembly of the primary autophagosomal membrane forms a complex of Beclin-1, the mammalian homolog of Atg6, with class III phosphatidylinositol 3-kinase (PI3K) that mediates the localization of autophagy-targeted proteins into the autophagic vesicles [4, 6]. Elongation of this isolated membrane is governed by two ubiquitin-like conjugation systems, Atg5-Atg12 complex, and microtubule-associated protein 1A/1B-light chain (LC3). Upon the activation of Atg12 by Atg7, Atg12 is transferred to Atg10 and is eventually conjugated to Atg5, which subsequently forms a complex with Atg16. Under basal conditions, LC3B exists in its cytosolic form LC3B-I. However, during autophagy LC3B-I is converted to its lipid-conjugated membrane-bound form LC3B-II, a process that is dependent on the Atg5-Atg12 complex throughout the course of membrane elongation. LC3B-II is associated with autophagosomes and promotes the formation of autophagic vacuoles [4, 6]. A brief summary of the autophagic process is shown in **Figure 1**.

2. Autophagy and cancer

The autophagic process of recycling damaged cytoplasmic organelles and proteins can serve as an alternative energy source during the period of metabolic stress and can play a role in the maintenance of homeostasis and viability. However, in cancer cells autophagy appears to play a dual role that can either allow prolonged cell survival or promote cell death [7, 8]. This dual role of autophagy in cancer, as both tumor suppressor and a protector of cancer cell survival, is not yet clearly understood. Autophagy dysregulation is observed in a wide spectrum of human cancers. For example, the altered expression of several autophagy proteins such as LC3B and Beclin-1 has been observed in brain, esophageal, breast, colon, gastric, liver, and pancreatic cancer as well as osteosarcoma and melanoma [9, 10]. Mutations of various autophagy-related genes have also been reported in gastrointestinal cancers [1]. It is clearly evident that greater understanding of the specific role of autophagy in the etiology of various types of cancer and at various stages of cancer progression will provide useful insights for the development of novel and more effective strategies for the prevention and treatment of cancer.

3. γ -Tocotrienol and cancer

Vitamin E is a generic term that represents eight chemically similar natural products that is subdivided into two subgroups classified as tocopherols and tocotrienols. The four tocopherol isoforms of vitamin E are more common and found in abundance in animal and vegetable oils. By contrast, the remaining four tocotrienol isoforms are quite rare, but found in high concentrations in palm oil [11, 12]. Both tocopherols and tocotrienols are characterized by a chromanol ring structure methylated to varying degrees at the 5, 7, and 8 positions to form eight different isoforms classified as α -, β -, γ -, and δ -tocopherols and α -, β -, γ -, and δ -tocotrienols. Attached to the chromanol ring is a long phytyl that is saturated on tocopherols and unsaturated on tocotrienols (**Figure 2**).

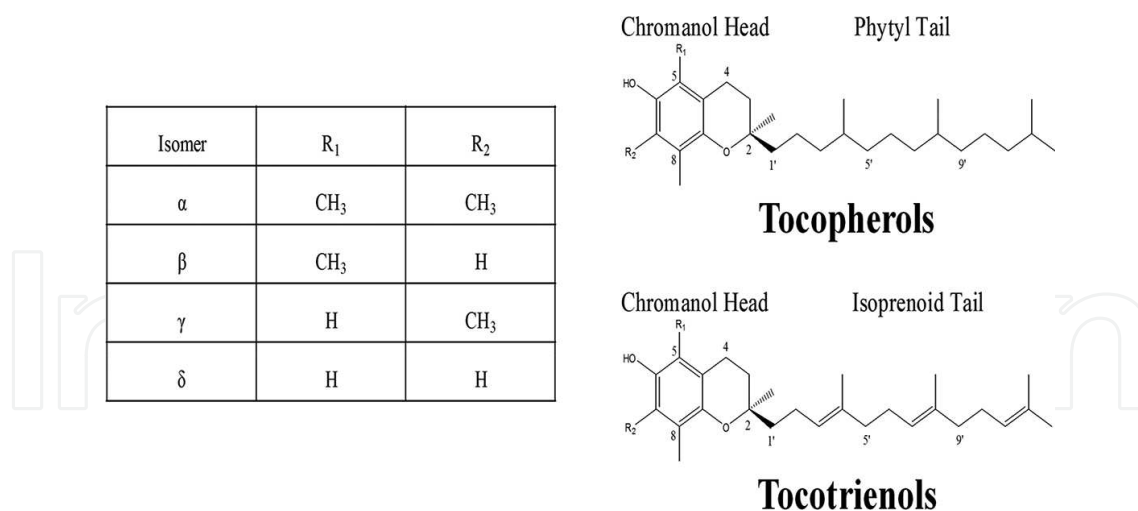


Figure 2. Chemical structure of tocopherols and tocotrienols.

Numerous *in vitro* and *in vivo* investigations have demonstrated the anticancer effects of tocotrienols [13–15]. Further studies showed that tocotrienols are preferentially and selectively taken up into mammary tumor cells as compared to tocopherols [16, 17]. Thus, tocotrienol displays potent anticancer activity at treatment doses that have little or no effect on the growth and viability of primary epithelial cells isolated from the mammary gland or immortalized mouse (CL-S1) and human (MCF-10A) normal mammary epithelial cell lines [16–18]. Additional reports have shown that the combined use of tocotrienols with other chemotherapeutic agents results in a synergistic anticancer response [19–23].

4. γ -Tocotrienol effects on mitogenic and apoptotic signaling

Various downstream signaling pathways are initiated by ligand-induced ErbB/HER receptor activation, including the mitogen-activated kinase (MAPK) and PI3K/Akt/mTOR, and activation of these cascades is associated with cellular growth, survival, and motility [24–26]. Specifically, studies have shown that tocotrienol inhibits PI3K/Akt-signaling pathways through the inhibition of EGF-dependent Akt phosphorylation (activation) in mammary cancer cells [27]. Moreover, inhibition of MAPK such as ERK, p38 MAPK, and activation of JNK is critical to the antiproliferative effects of tocotrienols [23, 28, 29].

Studies conducted by Wali et al. [30] were the first to demonstrate that treatment with 15–40 μ M of γ -tocotrienol induced mouse +SA mammary tumor cell death in a dose-dependent manner. Specifically, γ -tocotrienol induced cytotoxicity in these cells as associated with an increase in poly (ADP-ribose) polymerase (PARP) cleavage, an established cellular marker for apoptosis, as well as increased signaling of the protein kinase-like endoplasmic reticulum (ER) kinase/eukaryotic translational initiation factor/activating transcription factor 4 (PERK/eIF2 α /ATF-4) pathway, a marker of endoplasmic reticulum stress. These studies also showed

that γ -tocotrienol treatment also induced a large increase in C/EBP homologous protein (CHOP) levels and tribbles 3 (TRB3) expression [30]. ER-stress response also cleaved caspase-12 (activated), which is responsible for the disruption of ER calcium homeostasis and the accumulation of excess proteins in ER, and thus initiating the apoptosis signaling was observed following cytotoxic treatment of γ -tocotrienol for 24 h [30]. Studies have also shown that γ -tocotrienol treatment led to apoptosis, necrosis, and autophagy in human prostate PC-3 and LNCap cancer cells, as it causes an increase in the accumulation of dihydrosphingosine and dihydroceramide, important sphingolipids in de novo biosynthesis pathway, but have no effects on ceramide or sphingosine [31]. Other studies have shown that the γ -tocotrienol-induced autophagy is associated with the inhibition of mTOR activation [32].

5. Oridonin and cancer

Oridonin (7,20-epoxy-ent-kauranes), a diterpenoid isolated from the Chinese medicinal herb *Rabdosia rubescens*, is shown in **Figure 3**. Oridonin has also been shown to display potent anticancer activity [33]. Oridonin treatment was found to significantly inhibit tumor growth and induced cancer cell death in vivo [34], and was effective in suppressing tumor development, growth, and progression [33]. The antitumor effects of oridonin appear to include the suppression of cell cycle progression and/or the initiation of apoptosis [35]. Experimental investigations showed that following oridonin treatment, murine melanoma K1735M2 cells [35], DU-145 cells [36], and L929 cells [37] showed cell cycle arrest in the G2/M phase. In addition, other investigators have found that oridonin induced cell cycle arrest in the G1/S phase and this was associated with a corresponding inhibition in cdc2 and cyclin B activation in MCF-7 breast cancer cells [38]. Furthermore, oridonin-induced autophagy has been shown to be a prerequisite for the initiation of apoptosis in breast cancer cells [39].

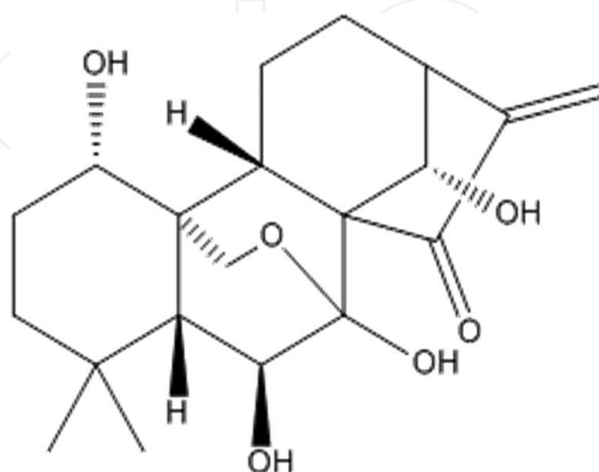


Figure 3. Chemical structure of oridonin.

6. Combination treatment of γ -tocotrienol with other chemotherapeutic agents

Most traditional cancer chemotherapies are not very selective and can cause damage to normal cells. It has become evident that a better approach is to use combination therapy that is more effective and produces less adverse side effects. Furthermore, the use of phytochemicals in the prevention and treatment of cancer has recently gained much interest, and combination therapies are attractive because an additive or synergistic therapeutic response can result. The rationale for using tocotrienols in combination therapy is based on the findings that a form of vitamin E has a broad range of anticancer actions and the principle that resistance to any single agent can be overcome by using multiple agents with complementary mechanisms of action [11, 12, 19–23, 40]. Previous studies have shown that combined low dose of γ -tocotrienol with other chemotherapeutic or phytochemical agents displays significantly enhanced anticancer effects, as compared to that of individual treatment alone [11]. It has also been shown that γ -tocotrienol synergizes with other phytochemical agents such as resveratrol to induce autophagy accompanied by the activation of Beclin and LC3-II and by decreasing mTOR signaling [32].

7. γ -Tocotrienol-induced autophagy in breast cancer cells

Previous investigations have shown that tocotrienol treatment induces autophagy in various cell types [31, 32, 41, 42]. Treatment with γ -tocotrienol was found to induce autophagy and apoptosis in rat pancreatic stellate [41] and prostate cancer cells [31], whereas other studies have shown that γ -tocotrienol treatment was cardioprotective and prevented apoptosis in ischemic cardiomyocytes [32]. However, the exact role of autophagy in mediating γ -tocotrienol-induced cytotoxicity has only recently been investigated [18]. In these studies, experiments were conducted to characterize γ -tocotrienol-induced autophagy in highly malignant mouse (+SA), and human estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) malignant mammary cancer cell lines. Results showed that γ -tocotrienol treatment significantly reduced cell viability in these breast cancer cell lines in a dose-responsive manner [18]. These same treatments also induced a corresponding increase in autophagy markers as determined by an increase in monodansylcadaverine (MDA) autofluorescence and flow cytometric analysis of positive acridine orange staining [18]. In addition, parallel studies determined that treatment with these same doses of γ -tocotrienol induced an increased conversion of microtubule-associated protein, 1A/1B-light chain 3, from its cytosolic form (LC3B-I) to its lipidated form (LC3B-II), the phosphatidylethanolamine-conjugated form associated with autophagosomes, and a corresponding increase in Beclin-1 and ATG6 in +SA, MCF-7, and MDA-MB-231 breast cancer cells.

These findings confirm and extend previous findings that showed that tocotrienol treatment promotes the conversion of LC3B-I to LC3B-II in other cell types [32, 41, 42]. By contrast, similar treatment with γ -tocotrienol was not found to increase autophagy marker expression in

immortalized mouse (CL-S1) and human (MCF10A) normal mammary epithelial cell lines, indicating that γ -tocotrienol displays selective action against cancer cells. Additional studies showed that γ -tocotrienol treatment also caused a reduction in PI3K/Akt/mTOR signaling and a corresponding increase in the Bax/Bcl-2 ratio, cleaved caspase-3, and cleaved PARP levels in these cancer cell lines, suggesting that γ -tocotrienol-induced autophagy may be involved in the initiation of apoptosis [18]. Since mTOR activity is directly associated with a suppression of autophagy [6] and Bcl-2 acts to suppress Beclin-1 levels [43], these findings indicate possible intracellular-signaling mechanisms that may be involved in mediating tocotrienol-induced autophagy and promote cytotoxicity in breast cancer cells. Selective effects of γ -tocotrienol on autophagy cellular markers in MDA-MB-231 human breast cancer cells are shown in **Figure 4**.

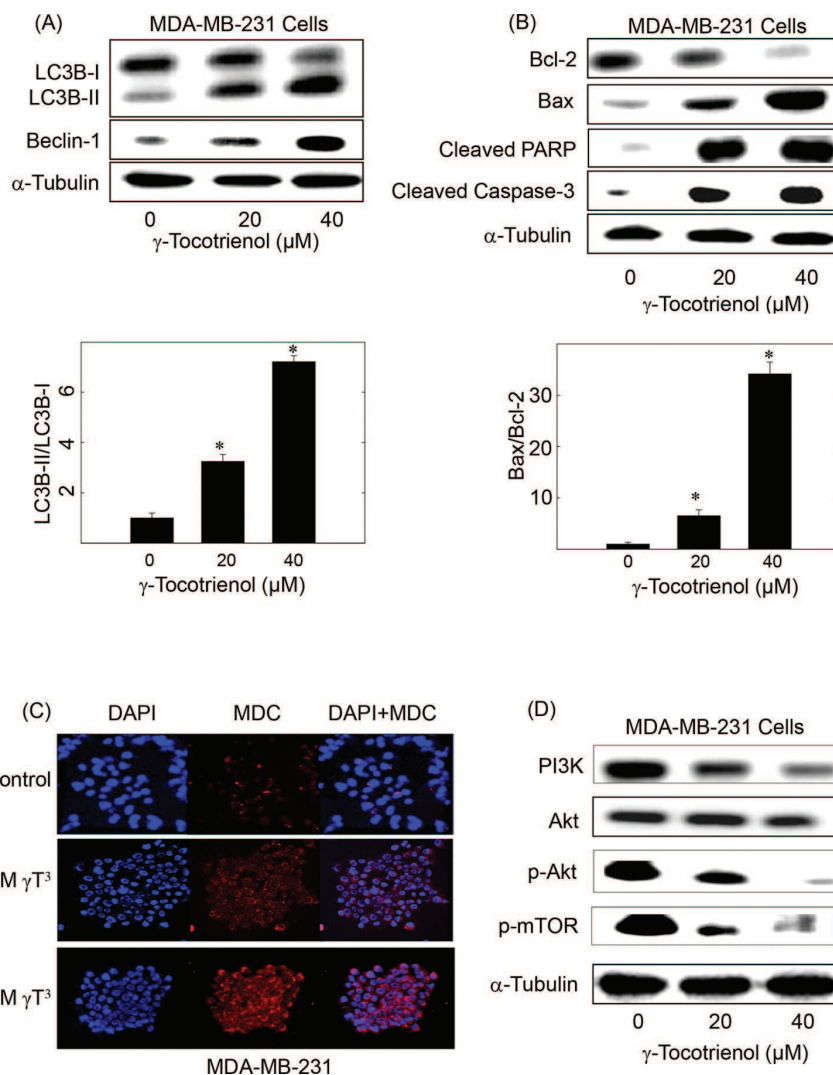


Figure 4. (A) Western blot analysis of γ -tocotrienol effects on the relative protein levels that serve as markers for autophagy in mammary cancer cells and scanning densitometric analysis of Western blots shown above. (B) Western blot analysis of γ -tocotrienol effects on the relative levels of apoptotic protein markers and scanning densitometric analysis of Western blots shown above. (C) Effects of γ -tocotrienol treatment on autophagic vacuoles MDC fluorescence intensity. (D) Western blot analysis of γ -tocotrienol effects on the relative levels of PI3K/Akt/mTOR signaling proteins. * $P < 0.05$ as compared to the vehicle-treated control group.

In summary, these initial studies provided evidence to support the suggestion that the cytotoxic effects of γ -tocotrienol are associated with the induction of autophagy in mouse and human breast cancer cells.

8. γ -Tocotrienol simultaneously induces autophagy and endoplasmic reticulum stress-mediated apoptosis in breast cancer cells

The endoplasmic reticulum is an intracellular organelle that is involved in protein synthesis, but during times of stress the ER plays an important role in programmed cell death [30]. ER-stress-mediated apoptosis is associated with an increased expression of several proteins including protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF-6), and inositol-requiring kinase 1 (IRE1) [44–46]. In addition, the increased expression of phosphorylated eukaryotic translational initiation factor 2 (eIF2 α), C/EBP homology protein (CHOP), tribbles 3, and ATF-4 also occurs during the initial phases of ER-stress-mediated apoptosis [30, 47–52].

Previous studies have shown that the anticancer effects of tocotrienols are associated with the induction of autophagy and endoplasmic reticulum-stress-mediated apoptosis [18, 30, 31, 53]. However, a direct causal relationship between tocotrienol-induced autophagy and ER stress had not yet been established. Recently, studies were conducted to characterize the interrelationship between γ -tocotrienol-induced cytotoxicity, autophagy, and ER-stress-mediated apoptosis in human breast cancer cells [54]. In these studies, γ -tocotrienol treatment causes an increase in the appearance of damaged and/or dying MCF-7 and MDA-MB-231 cancer cells together with a corresponding increase in the appearance of large autophagic vacuoles as visualized by Giemsa staining, a large increase in positive MDC fluorescent staining, a positive marker for autophagic vacuole formation [2, 55], and a large increase in positive LC3B fluorescent staining, a marker for autophagosomes [2, 55] in these same cells [54]. In addition, transfection with small interfering RNA (siRNA) targeting Beclin-1 prior to γ -tocotrienol treatment resulted in a modest but significant reduction in γ -tocotrienol-induced cytotoxicity as compared to cells transfected with scrambled siRNA (negative control) and then treated with γ -tocotrienol, and these effects were correlated with a corresponding large increase in Beclin-1 levels and LC3B-II/LC3B-I ratio [54].

Subsequent studies investigated the effects of γ -tocotrienol on the activation of stress-signaling pathways in these same MCF-7 and MDA-MB-231 breast cancer cells. Results showed that γ -tocotrienol induced an increase in the activation of p38 and JNK1/2, and simultaneous decrease in Erk1/2 signaling. This same treatment also induced a reduction in Bcl-2 (antiapoptotic), and an increase in Bax (proapoptotic), cleaved caspase-3 (activated), and cleaved-PARP (activated) protein levels in these breast cancer cells [54]. In addition, γ -tocotrienol treatment significantly increases the ratio of Bax/Bcl-2, and induced a time-dependent increase in the relative levels of ER-stress markers including Bip, IRE1, phosphorylated PERK (activated), phosphorylated eIF2 α (inactivated), ATF-4, CHOP, and TRB3 in MCF-7 and MDA-MB-231 breast cancer cells [54]. Combined treatment with the pan caspase inhibitor, zVADfmk, with γ -tocotrienol

resulted in a complete blockade of γ -tocotrienol-induced cytotoxicity in these cells [54]. During this same time period, γ -tocotrienol caused a time-dependent decrease in mitogenic Erk and Akt signaling, a corresponding increase in stress-dependent p38 and JNK activation [54].

Since autophagy may have a dual function in cancer that can either promote or suppress tumor cell survival [3, 4, 9, 10, 56], it is unclear whether autophagic activity in dying cancer cells is reflective of a compensatory mechanism that is trying to prevent death or is directly involved in promoting cell destruction. Previous studies have clearly demonstrated that γ -tocotrienol-induced cytotoxicity is not dependent on estrogen receptor status of breast cancer cells [57, 58]. Although it remains unclear why the γ -tocotrienol appears to selectively target cancer cells and not normal cells [14, 18], the possibility exists that noncancerous cells possess specific compensatory mechanisms that provide protection against γ -tocotrienol-induced ER-stress-mediated apoptosis and autophagy, and these self-survival mechanisms may be dysfunctional in malignant cells.

In summary, results obtained in the studies described above strongly suggest that γ -tocotrienol-induced breast cancer cell death is intimately related to the simultaneous initiation of autophagy and ER-stress-mediated apoptosis. This suggestion is further evidenced by the finding that pretreatment with agents that block the induction of autophagy resulted in a suppression in γ -tocotrienol-induced apoptosis. Taken together, these data indicate that γ -tocotrienol-induced autophagy and ER stress act concurrently to enhance the self-destruction of human breast cancer cells. The intracellular mechanism by which γ -tocotrienol-induced autophagy and ER stress initiate breast cancer-programmed cell death is shown as a schematic representation in **Figure 5**.

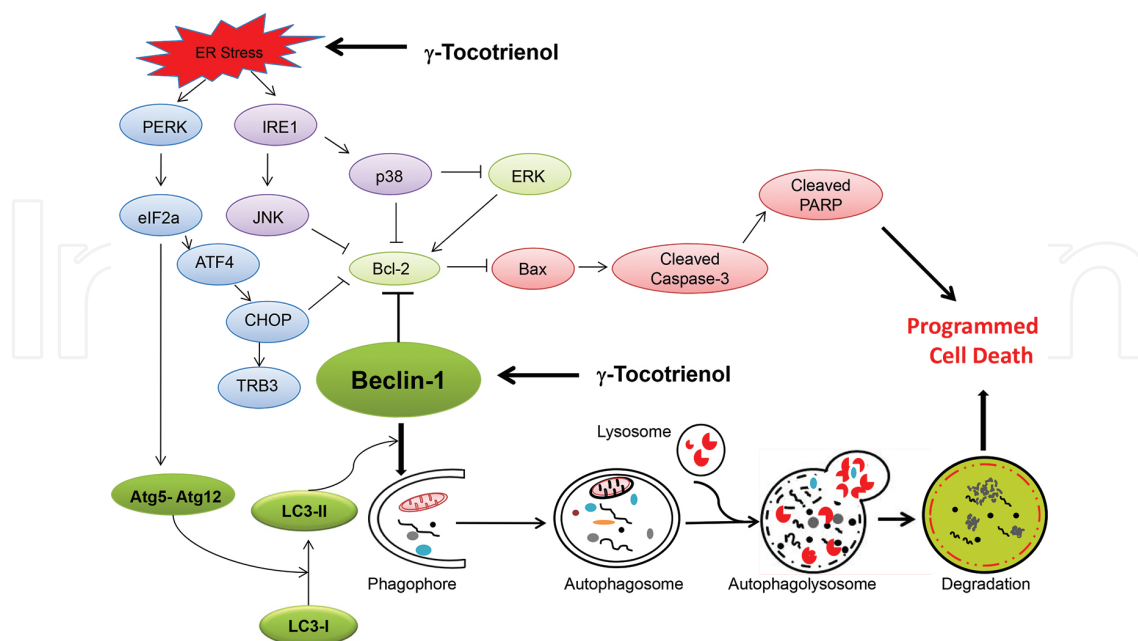


Figure 5. Schematic representation of the proposed molecular mechanism mediating γ -tocotrienol concurrent induction of autophagy and ER-stress-mediated apoptosis in breast cancer cells.

9. Synergistic anticancer effects of combined γ -tocotrienol and oridonin treatment

A great deal of interest has recently been generated in the development of novel therapies that target specific signaling pathways associated with neoplastic transformation, growth, and progression, because not only do these therapies provide enhanced anticancer efficacy, they also display significant less adverse or toxic effects on normal tissue. Phytochemicals appear to have great potential in this area not only for use as anticancer agents alone but also for use as potent supplemental agents that when combined with traditional anticancer therapies provide a synergistic therapeutic response [19, 59, 60]. γ -Tocotrienol displays potent antiproliferative, autophagic, and apoptotic activity against cancer cells at treatment doses that have little or no effect on normal cell growth and viability [12, 54]. Oridonin is another such phytochemical isolated from the herb *R. rubescens*, which displays potent anticancer activity against a wide range of cancer cell types [61–63]. Furthermore, oridonin-induced autophagy has been shown to be a prerequisite for the initiation of apoptosis in breast cancer cells [39]. Based on findings in the current literature, γ -tocotrienol-induced autophagy in breast cancer cells is associated with a reduction in mitogen-dependent PI3K/Akt/mTOR signaling and Bcl-2 expression [18]. These effects were also found to be directly related to an increase in Beclin-1 levels and the conversion of LC3B-I to LC3B-II in mouse and human breast cancer cell lines [18]. By contrast, oridonin-induced autophagy was shown to be directly related to a decrease in MAPK signaling and an increase in JNK- and p38-stress pathway signaling [39]. Combination therapy has also been shown to provide therapeutic advantages over monotherapy because a synergistic response is often observed using very low treatment doses and thereby reduce the emergence of toxic side effects [11].

Recent studies have also showed that combined treatment of subeffective doses of γ -tocotrienol and oridonin resulted in a significantly greater reduction in mammary tumor cell viability as compared to cells treated with either drug along [64]. Isobologram analysis of combination treatment with γ -tocotrienol and oridonin determined that these effects were synergistic. By contrast, similar combination treatment had no effect on the viability of normal mammary epithelial cells [64]. In addition, combined therapy significantly increased the conversion of LC3B-I to LC3B-II, as well as the expression of Beclin-1, Atg3, Atg7, Atg5-Atg12, LAMP-1, and cathepsin-D, established cellular markers of autophagy [64]. Furthermore, pretreatment with 3-methyladenine or bafilomycin A1, agents that prevent the induction of autophagy, blocked these effects induced by combined treatment with γ -tocotrienol and oridonin [64]. Additional studies showed that combination treatment with these phytochemicals also induced a large suppression in Akt/mTOR mitogenic signaling and corresponding increase in the levels of apoptotic cellular marker including cleaved caspase-3 and PARP, and Bax/Bcl-2 ratio in these same mammary tumor cells [64].

In summary, findings from these studies demonstrate that combined low-dose treatment of γ -tocotrienol and oridonin acts synergistically to induce autophagy and apoptosis in mammary tumor cells. Since these effects were associated with a large reduction in PI3K/Akt/mTOR signaling, these findings suggest that the combined use of the phytochemicals γ -tocotrienol

and oridonin may provide some benefit as supplemental or adjuvant therapy in the treatment of breast cancer.

10. Conclusion

Experimental data summarized above in this review provide convincing evidence that the anticancer effects of γ -tocotrienol are directly associated with the simultaneous initiation of autophagy and ER-stress-mediated apoptosis in breast cancer cells. This suggestion is further supported by the finding that chemical-induced blockade of γ -tocotrienol-induced autophagy significantly reduces γ -tocotrienol-induced apoptosis and cell death. These findings also show that γ -tocotrienol-induced autophagy is directly associated with a significant reduction in PI3K/Akt/mTOR signaling and corresponding increase in intracellular levels of Beclin-1 and conversion of LC3B-I to LC3B-II in these breast cancer cells. Furthermore, combination treatment with subeffective doses of γ -tocotrienol and oridonin acts synergistically in promoting the initiation of autophagy and apoptosis, indicating that the combined use of these natural phytochemicals may have value in the treatment of breast cancer in women.

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