Autophagy Inhibitor 3-Methyladenine Potentiates Apoptosis induced by Dietary Tocotrienols in Breast Cancer Cells

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Abstract

Introduction—Tocomin® represents commercially available mixture of naturally occurring tocotrienols (T3s) and tocopherol (Ts) extracted from palm oil/palm fruits that possess powerful antioxidant, anticancer, neuro/cardioprotective and cholesterol lowering properties. Cellular autophagy represents a defense mechanism against oxidative stress and several anticancer compounds. Recently, we reported that tocotrienols induce apoptosis and endoplasmic reticulum stress in breast cancer cells.

Methodology—We studied the effects of Tocomin® on MCF-7 and MDA-MB 231 breast cancer cells and non-tumor MCF-10A cells.

Results—Tocomin® inhibited cell proliferation and induced apoptosis in both MCF-7 and MDA-MB 231 breast cancer cell lines without affecting the viability of MCF-10A cells. We also showed that Tocomin® negatively modulates PI3K and mTOR pathways and induces cytoprotective autophagic response in triple negative MDA-MB 231 cells. Lastly, we demonstrate that autophagy inhibitor 3-methyladenine (3-MA) potentiated the apoptosis induced by Tocomin® in MDA-MB 231 cells.

Conclusion—Together our data indicates anticancer effects of Tocomin® in breast cancer cells which is potentiated by the autophagy inhibitor 3-MA.

Keywords

Vitamin E; Tocomin®; Tocotrienols; Breast Cancer; Apoptosis; Autophagy

Introduction

Vitamin E is composed of Tocopherols (Ts) and Tocotrienols (T3s) that have been shown to possess anti-cancer properties. Tocomin® represents commercially available mixture of

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Conflicts of Interest
The authors have no potential conflicts of interest

naturally occurring tocotrienols (78%) and tocopherols (22%) extracted from palm oil/palm fruits. It also contains other phytonutrients such as plant squalene, phytosterols, co-enzyme Q10 and mixed carotenoids extracted along with tocotrienols from palm fruits. Palm oil is predominantly rich in tocotrienols and has been demonstrated to possess more powerful antioxidant, anticancer, neuro/cardioprotective and cholesterol lowering properties than tocopherols [1–3]. Both tocopherols and Tocotrienols exist as four isoforms each (α, β, γ and δ). The accumulation of T3s in the cells is much greater than tocopherols and might be one of the reasons of a more significant physiological effects of tocotrienols than tocopherols [4].

T3s have been shown to inhibit the growth of various cancer cells including breast cancer without affecting the growth of normal cells [5–8]. T3s have been evaluated in vitro and in vivo as powerful cancer chemotherapeutic/preventive agents, yet their exact mechanisms of action on cell death and other inhibitory pathways are unknown [9,10,7,11,12]. Various mechanisms including blocking oxidative stress or radiation-induced DNA damage [7,13], modulation of immune response [14,15], suppression of multiple oncogenic signaling molecules and pathways such as PI3/AKT/β-catenin, NF-κB, ERK and cyclinD1 [16–21] and ceramide synthesis [22] have been suggested. Also, studies have shown that tocotrienols inhibit cell migration and invasion by modulating matrix metalloproteinases and their inhibitors [23] as well as negatively modulate VEGF dependent angiogenesis [24]. Tocotrienols exhibit cell inhibitory effects in breast cancer cell lines irrespective of their ER status, gene expression profiling in estrogen receptor (ER) positive, p53 wild type MCF-7 and ER negative, p53 mutant MDA-MB 231 cells treated with tocotrienol rich fraction (TRF) of palm oil suggested different mechanisms in the two cell lines [25]. Other mechanisms including activation of proapoptotic pathways including caspase-8 activation and mitochondrial dependency modulation of p53, Bax/Bcl2 [26,17,27,28] have been reported. Recent studies from our laboratory have suggested the role of ATF3 in the apoptosis induced by γ-T3 [29]. Also, we demonstrated the modulation PERK and IRE1α dependent endoplasmic reticulum-stress (ER-stress) and unfolded protein response (UPR) related pathways in MCF-7 and MDA-MB 231 cells when treated with γ-T3 [29]. ER-stress activates unfolded protein response (UPR) that can reestablish endoplasmic reticulum homeostasis through autophagy; however, persistent UPR can also lead to apoptosis. We demonstrated earlier that γ-T3 activates PERK signaling, which has also been shown to induce autophagy as a protective response to cellular insults, such as hypoxia and nutrient deprivation [30–32,29]. Similarly, IRE1α has also been implicated in autophagic response [33,34,31]. In the present study we used commercially available Tocomin® as a source of naturally occurring dietary tocotrienols and studied its effects on inducing autophagy and apoptosis. Further, we used 3-Methyladenine (3-MA), a widely used autophagy inhibitor to study whether combined treatment of 3-MA with Tocomin® modulates apoptosis in breast cancer cells.

1. Materials and Methods

2.1. Cell Culture and Media

Human breast cancer cells (MCF-7 and MDA-MB 231) and human nontransformed mammary epithelial cells (MCF-10A) were obtained from Lombardi Comprehensive Cancer Center cell repository and cultured as recommended.

2.2. Chemicals, Reagents and Antibodies

Tocomin® 50% (here after used as Tocomin®) is a mixture of natural tocotrienols (78%) and Tocopherols (22%), gifted by Carotech International (Edison, NJ) and dissolved in DMSO. WST-1 reagent used for cytotoxicity assay and protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN); 3-Methyladenine or 3-MA (Sigma, St. Louis, MO). ECL Plus Western blotting detection system (GE Healthcare, Piscataway, NJ); and Coomassie protein assay reagent (BioRad, Hercules, CA). All the antibodies were from Cell Signaling Technology Inc., Danvers, MA. Secondary antibodies conjugated with horseradish peroxidase included goat anti-mouse IgG, goat anti-rabbit IgG and rabbit anti-goat IgG (Jackson ImmunoResearch, West Grove, PA).

2.3. Cell Viability and Proliferation Assay

Effects of Tocomin® on cell viability and proliferation were determined as described earlier [29] using a cell viability detection kit (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate, WST-1) according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

2.4. MDC staining

Monodansylcadaverine (MDC) staining was used as a marker of autophagic vacuoles and lysosomes using Autophagic/cytotoxicity dual staining kit (Cayman Chemical Company, Ann Arbor, MI). Autophagic vacuole staining intensity was measured using an excitation wavelength of 335nm and an emission wavelength of 512nm following the manufacturer's instructions.

2.5. Western Blotting

Immunoblotting was performed essentially as described previously [29]. Each experiment was performed at least 2 times. Representative Western blots are shown.

2.6. Statistical Analyses

Cell proliferation experiments were performed in 6 replicates. Student's T test was used to analyze treated vs. untreated cells. Results were expressed as averages ± SD. $P < 0.05$ was considered significant.

3. Results

3.1. Dietary tocotrienols Tocomin® inhibit the proliferation of human breast cancer cells

We compared the effects of Tocomin® on the viability of MCF-10A mammary epithelial cells; and MCF-7 and MDA-MB 231 breast cancer cells using WST-1 assay. As shown in Figure 1, cell viability was significantly reduced in cells treated with 80μg/ml and 100μg/ml Tocomin® as compared to the vehicle treated control cells after 24h in both the cell lines. No significant effect on cell viability was observed in MCF-10A cells. Three independent batches of Tocomin® were used with similar results.

3.2. Tocomin® induces apoptosis

Next we studied the cell death mechanism and whether this mixture of dietary tocotrienols Tocomin® can induce apoptosis. We treated MCF-7 and MDA-MB 231 cells with Tocomin® at various concentrations ranging from 10μg/ml to 80μg/ml and analyzed the cleavage of PARP and caspases as apoptotic markers. In MDA-MB 231 cells we assessed the cleavage of terminal caspase, caspase-3. MCF-7 cells are deficient in caspase-3, thus we analyzed caspase-7 cleavage. Western blotting analysis demonstrated PARP cleavage as indicator of apoptosis in both the cell lines. Also, we observed caspase-7 and caspase-3 cleavage starting at 40μg/ml and 80μg/ml Tocomin® in MCF-7 and MDA-MB 231 respectively after 24h treatment. The results have shown that Tocomin® induces apoptosis in MCF-7 and MDA-MB 231 breast cancer cells after 24h of treatment (Figure 2).

3.3. Tocomin® induces autophagic response in MDA-MB 231 cells

Autophagy provides certain advantage to tumor cells and an autophagic response has been shown to be associated with almost all anti-cancer regimens as tumor cell-intrinsic, resistance mechanism [35–38]. Paradoxically, Autophagy is also induced by dietary compounds with cancer chemo preventative properties and an important role of autophagy has been suggested in chemoprevention of cancer [34,37,31]. Autophagic response also precedes an apoptotic response in certain cases of autophagic cell death. To obtain clues as to the mechanism by which Tocomin® induces apoptosis and whether, an autophagic response is part of Tocomin® induced cell signaling, we performed MDC fluorescent staining to measure autophagy in response to Tocomin®. MCF-7 and MDA-MB 231 cells were treated with Tocomin® ranging from 10–100 μg/ml in a 96-well plate. Cells were incubated with the autofluorescent drug Monodansylcadaverine (MDC), a specific autphagosome marker to detect autophagy. Figure 3A revealed that Tocomin® increased MDC fluorescence intensity only in MDA-MB 231 but not in MCF-7, cells indicating that Tocomin® causes an increase in autophagy in MDA-MB 231 cells but not in MCF-7 cells. Furthermore, we also confirmed the results by detecting the LC3β-II conversion by Western blotting. Both the cell lines were treated with indicated concentrations of Tocomin® from 10 to 80μg/ml for 24h followed by Western blotting with antibodies to the autophagic marker LC3β. As shown in Figure 3B, LC3β-I to II conversion was observed starting at 40μg/ml in MDA-MB 231 cells. No changes in LC3β expression was observed in MCF-7 cells (data not shown). 3-MA is a widely used autophagy inhibitor that inhibits class III phosphoinositide 3-kinase (PI3K). We examined whether 3-MA can inhibit the autophagy induced by

Tocomin®. As shown in figure 3C, co-treatment with 3-MA inhibited the autophagy induced by Tocomin® in MDA-MB 231 cells.

3.4. Tocomin® negatively modulates mTOR pathway

To investigate the mechanisms of Tocomin® induced autophagy, we studied the effects of Tocomin® on components of the mTOR pathway including the PI3 kinase class I that regulates mTOR signaling. The PI3K class I is mainly activated via the insulin receptor leading to the activation of AKT and mTOR complex 1 leading to the suppression of autophagy. As shown in Figure 4, both p85 and 110α subunits were downregulated after Tocomin® treatment. Furthermore, the expression levels of mTOR and Raptor were also downregulated as the concentration of Tocomin® was increased.

3.5. 3-MA modulates Tocomin® induced apoptosis

Previous studies have shown that during drug-induced protective autophagy, inhibition of the autophagy often leads to enhanced apoptotic response. We reasoned that if in our case, the autophagy is a resistance mechanism to Tocomin® induced apoptosis, we should also get a similar effect of autophagy inhibition on Tocomin® induced apoptosis. To test this hypothesis, we used 3-MA as an inhibitor of autophagy. MDA-MB 231 cells were starved overnight followed by treatment with 5mM 3-MA for 30 minutes before the combined treatment with sub-effective dose of 20μg/ml Tocomin® for 1, 6 and 24 h. As shown in figure 5, treatment with 3-MA and Tocomin® exhibited an increased apoptotic response as evident by PARP cleavage starting very early at 1h of the combined treatment with profound synergistic apoptotic effect evident at 24h. The results indicated that 3-MA mediated inhibition of autophagy modulates Tocomin® induced apoptosis.

4. Discussion

Vitamin E is composed of tocopherols and Tocotrienols which are two structurally similar compounds with diverse physiological functions. Much of the studies on the effects of Vitamin E have been performed predominantly on tocopherols. Recently several studies have indicated a much prominent role of Tocotrienols in human health including their compelling anti-cancer properties. Studies on purified Tocotrienols have shown that in addition to being pro-apoptotic, T3s also inhibit epithelial mesenchymal transition, angiogenesis and exhibit chemosensitizing as well as anti-cancer stem cell properties [8]. Earlier studies in our lab have shown that γ-T3 induced apoptosis is dependent on ATF3 and is also associated with unfolded protein response leading to endoplasmic reticulum stress (ER-stress) [29]. Autophagy is often activated as a cell survival mechanism followed by ER-stress and UPR [31] with its inhibition leading to sensitization to chemotherapeutic drugs [39–41,36]. In the present study we utilized Tocomin® as a source of primarily dietary tocotrienols (T3s) and studied its effects on MDA-MB 231 and MCF-7 breast cancer cells in inducing apoptosis and autophagy. Studies from our lab and others performed on purified tocotrienols and tocotrienol rich fraction from palm oil have demonstrated their apoptotic effects on various cancer cells without affecting non-tumor cells [8,28,25,1,29]. In a recent study, diet supplementation of tocotrienols to AIN93G-based diet for 13 weeks did not show any side effects in Fisher 344/slc rats [42]. In this study, we did not observe any cell-death

with Tocomin® in non-cancerous MCF-10A cells (Figure 1) suggesting a cancer-cell specific response. Here we demonstrate that Tocomin® inhibits cell proliferation and induces apoptosis in breast cancer cells regardless of their ER and p53 status. ER status independent apoptotic response in breast cancer cells by Vitamin E compounds have also been demonstrated in other studies [8,43,44]. Next we studied autophagic response of Tocomin®. In triple negative MDA-MB 231 cells we noticed a profound induction of autophagy as evident by MDC staining and LC3B immunoblotting (Figure 3). Interestingly, we did not observe any autophagy in MCF-7 cells when treated with Tocomin®. Bioavailability of vitamin E compounds vary for its different isoforms and mode of delivery [45]. Bioavailability of tocotrienols, tocotrienol rich fraction (TRF) alone and as inclusion with γ-cyclodextrins and other carriers has been studied [45–47]. Miyoshi eta al., reported the Cmax and AUC values in mice receiving TRF has ranged from 7.9±3.3 μM and 47.7±14.6 μM*h respectively [45]. The concentrations were modestly increase by 1.4 folds when TRF was delivered with γ-cyclodextrins (Cmax=11.5±4.5 μM and AUC=68.1±18.3 μM*h). The TRF exposure as reported corresponds to the Tocomin® concentrations used in this study emphasizing its physiological relevance. Chemopreventive and chemotherapeutic agents (including those that induce ER-stress and UPR) are known to induce autophagy in cancer cells, probably, initially as a protective response maintaining cellular homeostasis and eventually as promoter of cell death. In case of ER-stress inducing compounds such as Tocotrienols, autophagy can help in cope with ER-stress by eliminating the unfolded protein aggregates or ER-stress-induced cell death [32,36,37]. Although, our earlier studies indicated ER stress response in both MCF-7 and MDA-MB 231 cells by purified γ-T3 [29], we did not observe any autophagy in MCF-7 cells that may be explained by difference between the two compounds (Tocomin® vs. purified γ-T3) or other genetic differences between the two cell lines that are important in regulating autophagy. To eliminate the possibility of batch specific effect, 3 independent batches of Tocomin® were tested for similar results. To gain insights into the mechanisms involved in inducing autophagy in MDA-MB 231 cells, we analyzed the expression of multiple signaling proteins known to regulate apoptosis and autophagy. Class I phosphoinositide 3-kinase (PI3K) pathway plays important role in the regulation of apoptosis and autophagy. PI3K is activated by growth receptor signaling at the plasma membrane and activates AKT activating mTOR pathway that inhibits autophagy [34,30,31]. The PI3K enzyme is composed of a regulatory p85 subunit and a catalytic p110 subunit. P85 binds, stabilizes and inhibits the p110 subunit. As shown in Figure 4, we observed downregulation of PI3 kinase subunits and mTOR components in cells treated with Tocomin®. Inhibition of PI3K (p85 and p110) along with mTOR and its binding partner Raptor by Tocomin® may contribute to its apoptotic and autophagic properties. The interaction of p85 and p110 is a complex process. Inhibition of p85 stabilizes p110 and contributes to cell survival. However, we also observed a downregulation of p110 that suggest and over all negative modulation of PI3K pathway by Tocomin® and may be responsible for inhibiting mTOR resulting in apoptosis as well as induction of autophagy. Autophagy induced by anticancer agents can be cytoprotective and the inhibition of such autophagic response has been shown to enhance cell death inducing properties of chemotherapeutic agents [37,36]. If autophagic response to Tocomin® is a cell survival response as it is in case of several anti-cancer therapies, we asked if MDA-MB 231 cells can be sensitized to Tocomin® by inhibiting autophagy. As shown in figure 5,

treatment with a well-known autophagy inhibitor 3-MA resulted in increased apoptosis in Tocomin® treated cells. 3-MA is a widely used inhibitor of autophagy that blocks the formation of autophagosome by modulating class III PI3K which is involved in the sequestration of cytoplasmic material during autophagy. In conclusion, although, further studies are warranted to further elucidate the mechanisms of action of Tocomin®, we demonstrate that Tocomin® exhibits anticancer effects in breast cancer cells and like several chemopreventive and chemotherapeutic agents induce cytoprotective autophagic response.

Acknowledgments

Abbreviations

T3s	Tocotrienols
Ts	Tocopherols
3-MA	3-Methyladenine
ER	Estrogen receptor
TRF	Tocotrienol rich fraction of palm oil
γ-T3	Gamma tocotrienol
ER-Stress	Endoplasmic reticulum-stress
UPR	Unfolded protein response
MDC	Monodansylcadaverine
PI3K	Phosphoinositide 3-kinase
mTOR	Mammalian Target of Rapamycin
LC3β	microtubule-associated protein 1light chain 3 beta

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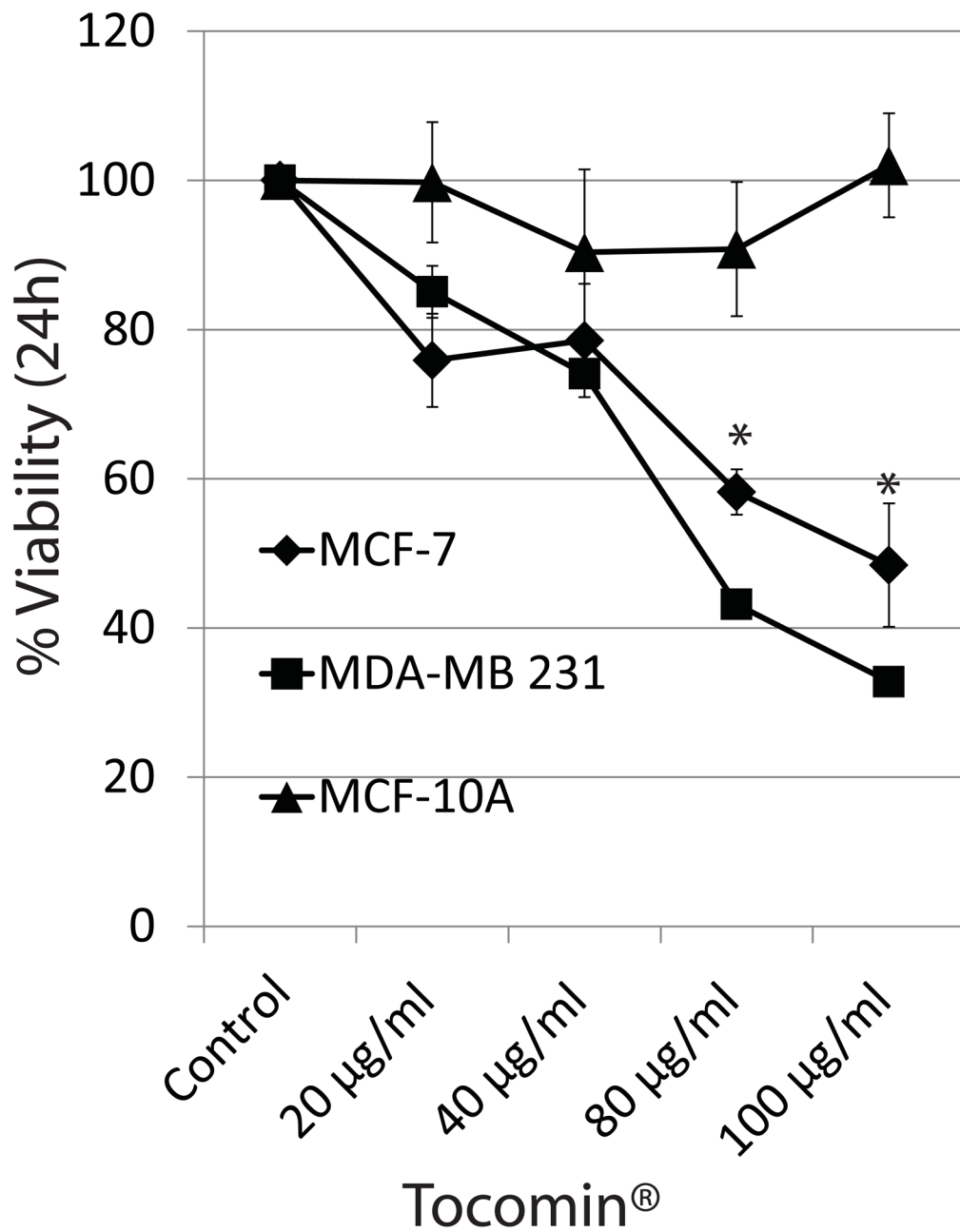
Fig. 1.
Tocomin® inhibits the proliferation of MCF-7 and MDA-MB 231 breast cancer cells. Cells were seeded at a density of 3000 cells/well in a 96 well plate and treated with DMSO control or indicated concentrations of Tocomin® for 24h. Cell proliferation was determined by WST-1 assay (Roche). A representative of 3 experiments performed in six replicates each is shown. P<0.05 (*) was considered significant.

MCF-7

| 0 | 10 | 20 | 40 | 80 | Tocomin® (µg/ml) |

Cleaved PARP

Cleaved Caspase 7

GAPDH

MDA-MB 231

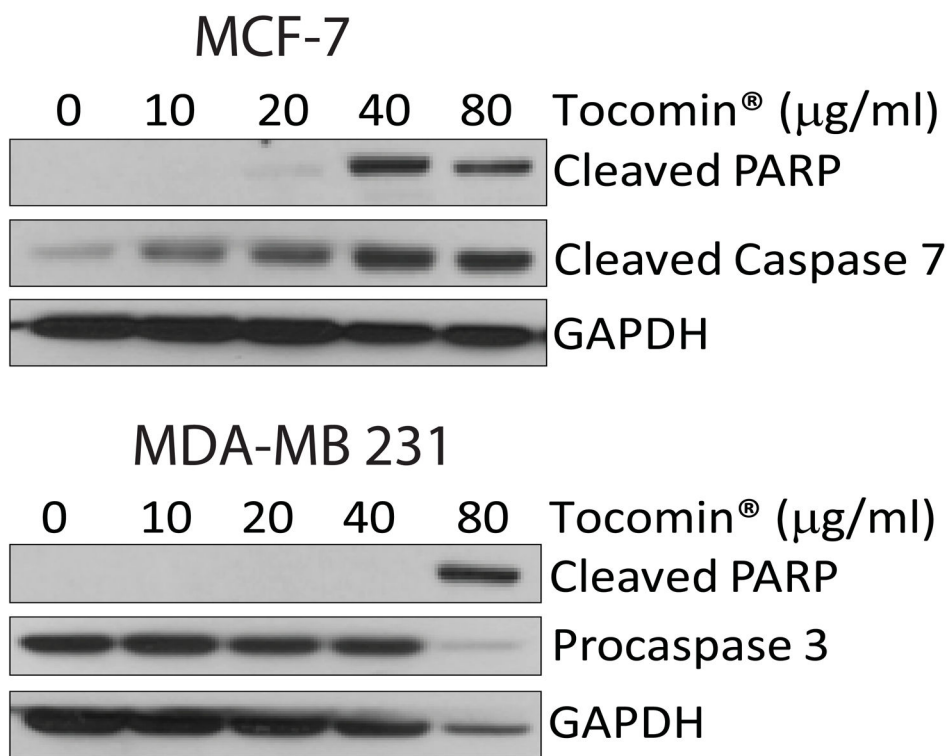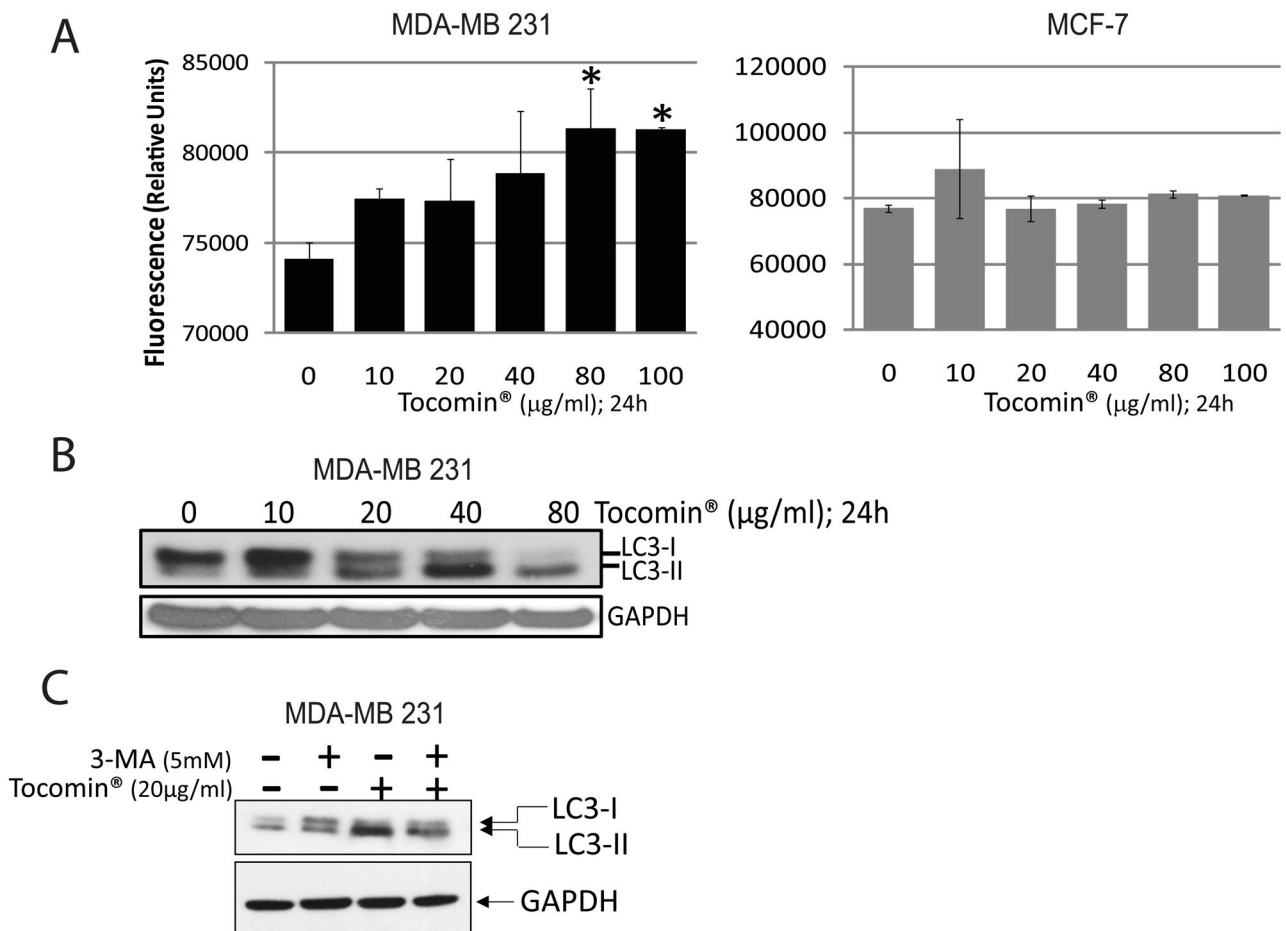| 0 | 10 | 20 | 40 | 80 | Tocomin® (µg/ml) |

Cleaved PARP

Procaspase 3

GAPDH

Fig. 2.
Tocomin® induces apoptosis in MCF-7 and MDA-MB 231 cells. Cells were treated with indicated concentrations of Tocomin® for 24h and the expression levels of cleaved-PARP, cleaved caspase-7 and pro-caspase-3 were determined by immunoblotting as an indicator of apoptosis. GAPDH was used as loading control.

Fig. 3.

Induction of autophagy by Tocomin® in MDA-MB 231 cells. A. Indicated cells were seeded at a density of 6000 cells/well in a 96 well plate and treated with DMSO control or indicated concentrations of Tocomin® for 24h. Fluorescent autophagic Monodansylcadaverine (MDC) staining (Cayman Chemical Company) was used as a marker of autophagic vacuoles. A representative of 3 experiments performed in six replicates each is shown. $P<0.05$ (*) was considered significant. Tocomin® induced autophagy was observed only in MDA-MB 231 cells (3A left panel) and not in MCF-7 cells (3A right panel). B. MDA-MB 231 cells were treated with indicated concentrations of Tocomin® for 24 h and autophagy was determined by Western Blotting using LC3 antibody; C. Pretreatment with 3-MA inhibits tocomin induced autophagy. Cells were treated with 3-MA for 30 minutes followed by treatment with indicated concentrations of Tocomin® for 24h. Autophagy was determined by Western Blotting using LC3 antibody. GAPDH was used as loading control.
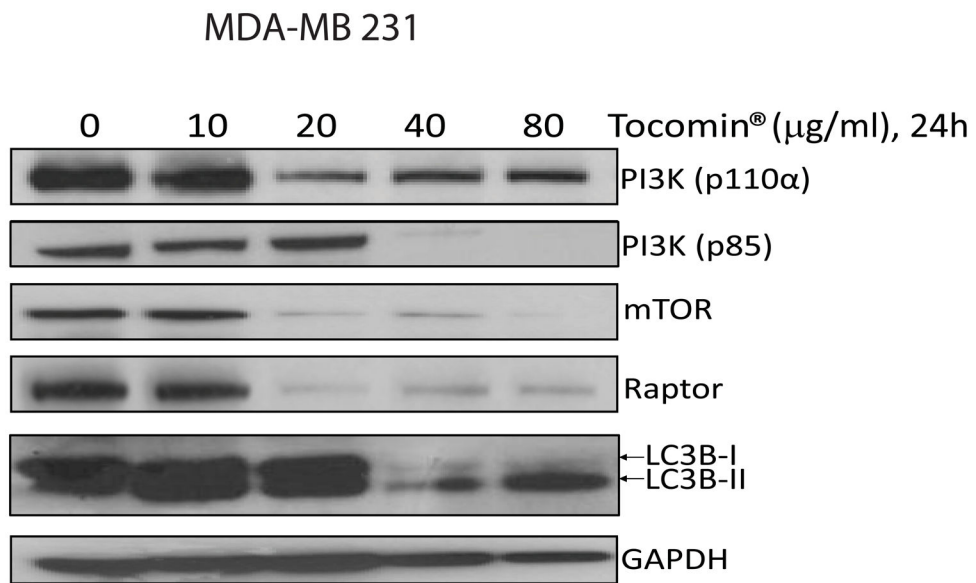
MDA-MB 231

Fig. 4.
Tocomin® modulates the expression of proteins involved in the PI3K/mTOR pathway. MDA-MB 231 Cells were treated with indicated concentrations of Tocomin® for 24h and the expression of indicated proteins was determined by immunoblotting. GAPDH was used as loading control.
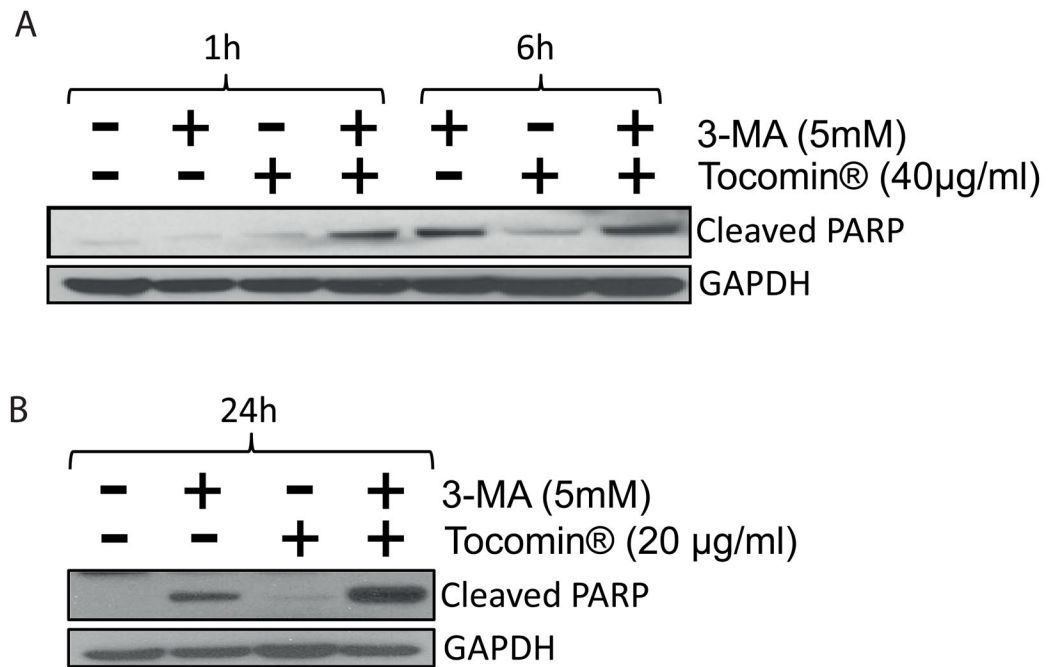
A

1h 6h

| − | + | − | + | + | − | + | 3-MA (5mM) |
| − | − | + | + | − | + | + | Tocomin® (40µg/ml) |

Cleaved PARP

GAPDH

B

24h

| − | + | − | + | 3-MA (5mM) |
| − | − | + | + | Tocomin® (20 µg/ml) |

Cleaved PARP

GAPDH

Fig. 5.
Pretreatment with 3-MA modulates Tocomin® induced apoptosis in MDA-MB 231 cells.
MDA-MB 231 cells were starved overnight and treated with 5mM 3-MA for 30 minutes
followed by 20µg/ml Tocomin® for 1, 6 and 24 h. Apoptosis was determined by detecting
PARP cleavage by immunoblotting. GAPDH was used as loading control.