Disruption of mitochondria during tocotrienol-induced apoptosis in MDA-MB-231 human breast cancer cells

By: Keiko Takahashi, George Loo

Takahashi, K. and Loo, G. (2004) Disruption of Mitochondria during Tocotrienol-Induced Apoptosis in MDA-MB-231 Human Breast Cancer Cells. Biochem. Pharmacol. 67, 315-324. DOI: 10.1016/j.bcp.2003.07.015

Abstract:

Tocotrienols, which are Vitamin E isoforms, are known to inhibit the growth of human breast cancer cells due partly to apoptosis. However, the characterization of tocotrienol-induced apoptosis is incomplete, particularly what happens during the initiation phase that precedes execution of the cells. The objective of this study was to clarify the apoptotic effects of tocotrienols, with especial emphasis in determining if the mitochondria-mediated death pathway is activated when human breast cancer cells are incubated with a specific tocotrienol isomer. During incubation with γ-tocotrienol, MDA-MB-231 human breast cancer cells showed membrane blebbing, and apoptotic bodies were present. Upon 4',6-diamidino-2-phenylindole staining of the cells, chromatin condensation and fragmentation were observed. Additionally, the annexin V-binding assay detected the translocation of membrane phospholipid during earlier analysis of the cells. Taken together, these results further establish that γ-tocotrienol can induce apoptosis in human breast cancer cells. To help elucidate how γ-tocotrienol induced the apoptosis, some important parameters related to the mitochondria-mediated death pathway were examined next. In γ-tocotrienol-treated cells, the mitochondria were disrupted. Collapse of the mitochondrial membrane potential was detected, and cytochrome c was released later from mitochondria. However, expression of Bax and Bcl-2 (mRNA and protein) did not change. Furthermore, poly-(ADP-ribose)-polymerase cleavage was not detected, suggesting that caspases were not involved in the γ-tocotrienol-induced apoptosis. These results imply that cytochrome c is not the critical protein released from mitochondria that triggers γ-tocotrienol-induced apoptosis in MDA-MB-231 cells.

Keywords:

Apoptosis; Cytochrome c; Mitochondria; Mitochondrial membrane potential; Mitochondrial permeability transition; Tocotrienols

Article:

1. INTRODUCTION

Tocotrienols are isoforms of Vitamin E and found abundantly in foods, such as palm oil and rice bran oil [1]. Like the related tocopherols, tocotrienols have substantial antioxidant activity [1,2] that may explain some, but not all, of their biological effects. Tocotrienols are thought to have health-promoting properties, including the capability to prevent cancer [3].

A few cellular studies have been conducted in characterizing the ability of tocotrienols to inhibit cancer cell proliferation. Tocotrienols inhibited the proliferation of human breast cancer cells [4] and did so irrespective of estrogen receptor status [5,6]. In these studies, the mechanism was not determined. It was unclear whether cell cycle arrest and/or cell death occurred. Later, however, it was reported that tocotrienols can induce apoptosis in human breast cancer cells [7], although the apoptotic events were not fully characterized. Interestingly, two studies [4,7] that compared the effects of tocopherols and tocotrienols on human breast cancer cells showed that

tocotrienols inhibited cell growth [4] and induced some signs of apoptosis [7], whereas α-tocopherol had no such effects. Hence, because the α-tocopherol was unable to induce apoptosis, the tocotrienols apparently killed the human breast cancer cells without relying on their antioxidant activity.

Presently, the death signaling events that are initiated as a result of incubating human breast cancer cells with tocotrienols are unknown. However, based on what is already generally known about apoptosis [8,9], it is possible that the caspases (aspartate-specific cysteine proteases) are activated as a pivotal event to execute the cells [10]. The main executioner is caspase-3, which either degrades or activates numerous important cellular proteins. For example, some substrates of caspase-3 include transcription factors, such as STAT1 [11], NF-κB [12], and SREBP [13], that are degraded upon enzyme catalysis. Activated caspase-3 also catalyzes the degradation of poly(ADP-ribose)-polymerase (PARP) [14] that is involved in DNA repair, Bcl-2 [15] that is a cell survival protein, Rb protein [16] that functions in cell cycle checkpoint regulation, and p21 [17] that promotes cell cycle arrest by inhibiting cyclin-dependent kinases. On the other hand, caspase-3 can activate a DNA-fragmenting endonuclease by degrading an inhibitory subunit of caspase-3 known as ICAD/DFF 45 [18]. There are two major apoptotic models or death signaling pathways that lead to the activation of caspase-3, namely, the cell surface death receptor pathway [9,10] and the mitochondria-mediated death pathway [8,10]. As such, it is conceivable that one of these pathways is involved in the induction of apoptosis when human breast cancer cells are incubated with tocotrienols.

In this study, we further characterized the apoptotic events induced by γ-tocotrienol and subsequently focused on the mitochondria-mediated death pathway to help clarify the cellular events leading to the execution phase of apoptosis. We examined some major steps that are associated with the mitochondria-mediated death pathway, such as reduction and collapse of mitochondrial membrane potential (MMP), release of cytochrome c, caspase-mediated proteolytic cleavage of PARP, and changes in Bax and Bcl-2 gene expression.

2. MATERIALS AND METHODS

2.1. Materials

MDA-MB-231 human breast cancer cells were obtained from ATCC. The γ-tocotrienol was a kind gift from Dr. Andreas Papas of Eastman Kodak. Cytochrome c protein standard was obtained from Sigma Chemical Co. Mouse anti-cytochrome c monoclonal antibody was purchased from BD Pharmingen. PARP protein standard was obtained from Biomol Research Laboratories Inc. Rabbit anti-PARP polyclonal antibody was purchased from Roche-Boehringer Mannheim. Rabbit anti-human Bax polyclonal antibody and rabbit anti-human Bcl-2 polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. Monoclonal anti-β-actin clone AC-15 (mouse IgG1 isotype) was purchased from Sigma. Annexin V-Alexa 488 was purchased from Molecular Probes Inc. The DePsipher™ Kit for detecting changes in MMP was purchased from Trevigen Inc. The Bax-α (human) gene-specific relative RT–PCR kit, QuantumRNA™ Classic 18S rRNA internal standard, and QuantumRNA™ β-actin internal standard were all obtained from Ambion Inc. Human Bcl-2 RT–PCR primer set was obtained from Continental Laboratory Products (CLP) Inc.

2.2. Cell culture

MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 media supplemented with 10% FBS, 1% penicillin/streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin), and 0.2% fungizone (0.54 μM/ L). The cells were cultured at 37° in a humidified incubator (100% air). The cells were subcultured every 4 days at a ratio of 1:20, and cell culture medium was renewed two to three times a week. For the experiments, the cells were incubated with γ-tocotrienol dissolved in DMSO (final concentration of DMSO in culture medium—0.1%) or with DMSO alone in the case of control cells, with more exact details given below.

2.3. Assessment of cell viability

MDA-MB-231 cells were seeded in 6-well microplates and grown until the confluency reached 70–80%. Then, the cells were incubated with 0–50 μg/mL γ-tocotrienol for 0–96 hr. After the cells were exposed to 0.4% (w/v)

trypan blue dye solution, the number of the cells not stained with trypan blue was counted as viable cells. The number of viable cells was expressed as a percentage of the total number of cells that were examined.

2.4. Detection of membrane phospholipid translocation

MDA-MB-231 cells (2.5 x 10^4 cells/mL) were seeded in 6-well microplates and allowed to proliferate until the confluency reached 70–80%. After the cells were incubated with 0 or 25 μg/mL γ-tocotrienol for 4 hr, the media were removed carefully, and 2 mL of annexin V-binding buffer was added to each well. Then, 200 μL of annexin V-Alexa 488 working solution was added to each well, and the cells were incubated for 15 min at room temperature. The cells were immediately examined with an Olympus IX-60 fluorescence microscope with SPOT digital camera to detect the presence of annexin V-Alexa 488 binding (green fluorescence) to the phosphatidylserine (PS) that translocated from the inner leaflet to outer leaflet of the membrane during the early stages of apoptosis.

2.5. Determination of chromatin condensation and fragmentation

MDA-MB-231 cells (2 x 10^4 cells/mL) were seeded in 6-well microplates and grown until the confluency reached 70–80%. After the cells were incubated with 0 or 25 μg/mL γ-tocotrienol for 0–96 hr, they were detached with a commercial non-enzymatic cell dissociation solution (Sigma), washed with PBS, and transferred to glass microscope slides using a Stat Spin Cytofuge. The slides were immersed in fixation solution (10% acetic acid in ethanol) for 30 min, washed with PBS, stained with 1 μg/mL 4', 6-diamidino-2-phenylindole (DAPI), and then examined by fluorescence microscopy to detect the presence of chromatin condensation/fragmentation as a late marker of apoptosis. Basically, 200 cells were randomly selected and scored each time. The cells that showed the late apoptotic feature were counted as a percentage of the total number of cells scored.

2.6. Detection of changes in MMP

MDA-MB-231 cells were seeded at 2.5 x 10^5 cells/10 mL in 10 cm petri dishes and grown until the confluency reached 70–80%. After the cells were incubated with 0 or 25 μg/mL γ-tocotrienol for 3 hr, they were stained with Trevigen's DePsipher™ solution (5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) according to the manufacturer's instructions. The stained cells were examined by fluorescence microscopy (Olympus BX-60 fluorescence microscope) and flow cytometry (BD FACSCalibur).

2.7. Preparation of whole cell lysates, mitochondrial and cytosolic fractions

The MDA-MB-231 cells were harvested after scraping and then centrifuged at 500 g for 5 min. The pellets were washed with Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS). To prepare whole cell lysates, the final pellets were resuspended in lysis buffer (1 M Tris–HCl, 2.5 M NaCl, 0.1% Nonidet P-40, 1% sodium deoxycholate, 10% SDS, 0.1 mg/mL PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.1 mM dithiothreitol (DTT), and 1% sodium orthovanadate) and left on ice for 30 min. After the cell suspensions were centrifuged at 5000 g for 5 min at 4°, the supernatants were collected and stored at —80°. To prepare mitochondrial and cytosolic fractions [19], the MDA-MB231 cells were washed with HBSS and resuspended in 700 μL of buffer A consisting of 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM Na-EGTA, 1.5 mM NaEDTA, 1 mM MgCl2, 1 mM DTT, and Complete Protease Inhibitor Cocktail (Roche-Boehringer Mannheim), pH 7.4, and incubated for 30 min on ice. After the cells were disrupted by 50 strokes with a glass Dounce homogenizer/B-type pestle, homogenates were centrifuged at 800 g for 10 min at 4 °. Supernatants were collected and further centrifuged at 22,000 g for 15 min at 4°, and the subsequent supernatants were stored as cytosolic extracts at —70°. The pellets were lysed further in buffer B (50 mM HEPES, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 2 mM DTT, and cocktail of protease inhibitors, pH 7.4) and incubated for 20 min on ice. After the cells were centrifuged at 22,000 g for 15 min, the supernatants were stored as mitochondrial extracts at —70°. The protein concentrations of the whole cell lysates as well as mitochondrial and cytosolic fractions were determined by the BCA protein assay according to the instructions from the manufacturer (Pierce Inc.).

2.8. Western immunoblotting analysis of cytochrome c, Bax, Bcl-2, and cleavage of PARP protein

Whole cell lysates (25 μg protein) were loaded in separate wells of Novex pre-cast NuPAGE mini-gels (4–12% Bis–Tris) for separation of Bax, Bcl-2, and PARP. Cytosolic and mitochondrial extracts (25 μg protein) were used for the analysis of cytochrome c. The gel-separated proteins were transferred to Hybond HCL nitrocellulose membrane (Amersham), which was then blocked with 3% non-fat milk in 0.1% Tween–PBS for 2 hr. The nitrocellulose membrane was probed with rabbit anti-human Bax, Bcl-2, and PARP polyclonal antibodies (1:5000) or mouse anti-human cytochrome c monoclonal antibody (1:2000) overnight. The membrane was subsequently treated for 1 hr with the secondary antibody, horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:600,000). After placing in a plastic pouch, the membrane was incubated with a chemiluminescent reagent (Pierce SuperSignal WestFemto Maximum Sensitivity Substrate) for 5 min and then examined on a Kodak Digital ScienceTM Image Station 440 CF for densitometric analysis. After densitometry, the nitrocellulose membranes were kept in the plastic pouch overnight. Then, they were removed, washed with 0.1% Tween–PBS, and reprobed overnight with mouse monoclonal anti-β-actin antibody (1:5000). After incubation for 1 hr with the secondary antibody, HRP-conjugated goat polyclonal anti-mouse IgG (1:600,000), the membrane was processed as before. The β-actin was used as a housekeeping protein to normalize the data for Bax and Bcl-2 protein in each sample.

2.9. Determination of Bax and Bcl-2 mRNA levels

Total RNA was isolated from the harvested cells by using a QIAGEN RNeasy Mini Kit according to the instructions provided by the manufacturer. Using a QIAGEN OneStep RT–PCR kit and gene-specific primers along with 18S rRNA or β-actin housekeeping gene/competimer sets (Ambion), Bax and Bcl-2 mRNA levels were determined by multiplex relative RT–PCR analysis following the recommendations of both manufacturers. Briefly, 40 μL of RT–PCR master mix containing RNase-free water, 5 x QIAGEN OneStep RT–PCR buffer, QIAGEN dNTP mix, QIAGEN OneStep RT–PCR enzyme mix, RNase inhibitor, and Bax or Bcl-2 primer mixture was added to each tube. Then, 6 μL of β-actin primer:competimer mix (2:8) for Bax or 18S rRNA primer:competimer mix (2:8) for Bcl-2 was added to each tube. Finally, 2 μg of template RNA in RNase-free water was added to each tube followed by mixing. The RT reaction was for 30 min at 50°, followed by 26 PCR cycles (denaturation at 94° for 30 s, annealing at 62° (for Bax) or 60° (for Bcl-2) for 30 s, extension at 72° for 1 min) and a final elongation at 72° at 10 min for both Bax and Bcl-2. The RT–PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining. The Bax primer set yielded a PCR product of 415 bp, while the P-actin primer set produced a product of 294 bp. The Bcl-2 primer set yielded a 293 bp product, while the 18S rRNA classic primer set produced a product of 495 bp.

2.10. Statistical analysis

Where appropriate, the data were expressed as mean ± standard error and analyzed by the one-way ANOVA Tukey HSD multiple comparison test using the SPSS v9.01 (SPSS Inc.). Differences were considered as significant at $P < 0:05$.

3. RESULTS

3.1. Morphological signs of apoptosis in y-tocotrienol-treated cells

To see if γ-tocotrienol altered cell morphology indicative of apoptosis, the MDA-MB-231 cells were examined by light microscopy (Fig. 1). After incubation for 4 hr with 25 μg/mL γ-tocotrienol, the morphology of the cells began changing. That is, the cells became round in appearance and membrane blebbing was seen. After 24 hr, apoptotic bodies and more cellular debris were apparent. To confirm that γ-tocotrienol killed the MDAMB-231 human breast cancer cells, trypan blue dye exclusion analysis was performed to assess viability of cells that were incubated for various times with increasing concentrations of γ-tocotrienol (Fig. 2). After 24 hr and beyond of being incubated with 10–50 μg/ml γ-tocotrienol, the number of viable cells started to become much noticeably lower, i.e. the number of cells actually dead started to increase.

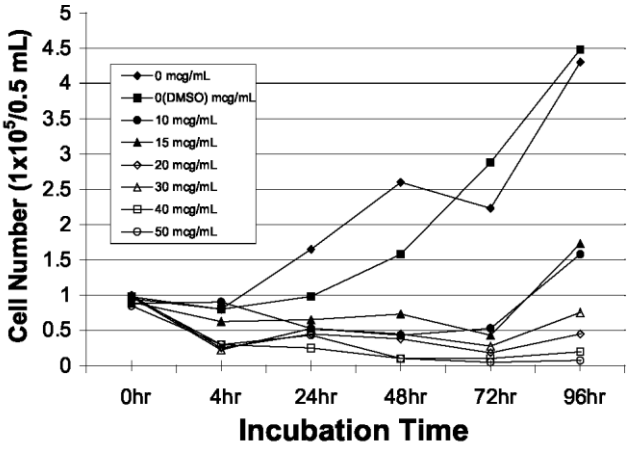

Fig. 2. Typical effect by γ-tocotrienol (γ-T3) in reducing the viability of MDA-MB-231 cells. Cells were incubated with 0–50 μg/mL γ-T3 for 0–96 hr. Cell viability was determined by counting the number of cells able to exclude trypan blue dye. Data are the average of two determinations.

3.2. Increased annexin V-Alexa 488 binding by y-tocotrienol-treated cells

The above results do not adequately establish the occurrence of apoptosis in γ-tocotrienol-treated MDAMB-231 cells. To detect a cardinal early feature of apoptosis, namely, translocation of PS from the inner to outer plasma membrane region, the more definitive annexin V-binding assay was performed on cells after being incubated with 25 μg/mL γ-tocotrienol for 4 hr. The PS that translocates to the outer region of the plasma membrane binds the annexin V conjugated to Alexa 488 fluor, which emits green fluorescence upon excitation [20]. Hence, by comparing the representative photos from fluorescence microscopy (Fig. 3A and B), it can be seen that a larger number of the γ-tocotrienol-treated cells (Fig. 3B) compared to untreated cells (Fig. 3A) bound annexin V-Alexa 488. This observation was typical of repeated, separate experiments with the results summarized in Fig. 3C.

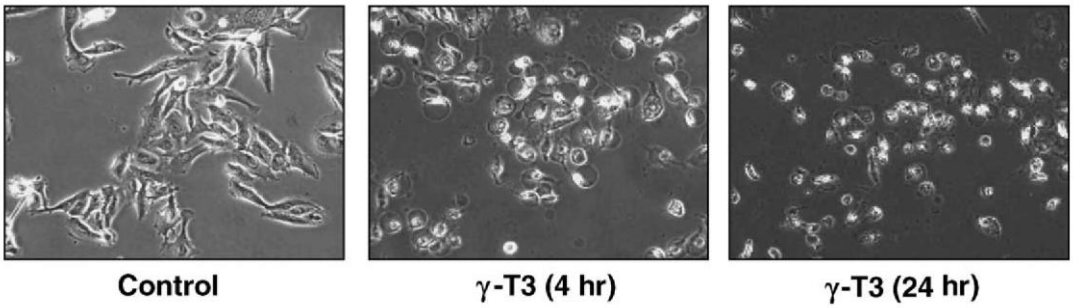

Fig. 1. Characteristic changes in the morphology of MDA-MB-231 cells caused by γ-tocotrienol (γ-T3). Cells were incubated with 25 μg/mL γ-T3 for either 4 or 24 hr. For comparison, control cells were incubated with the solvent (DMSO) for γ-T3. Afterwards, the cells were examined by light microscopy.

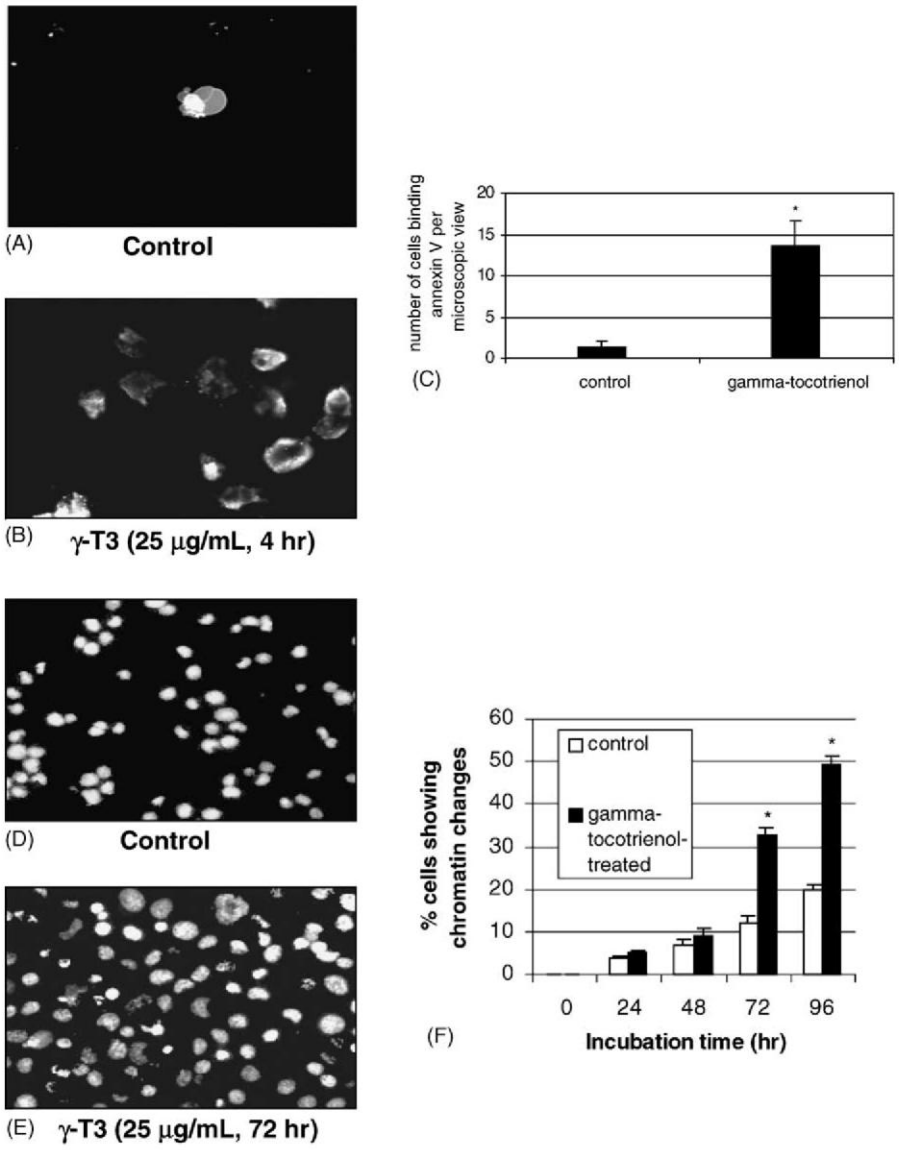

Fig. 3. Greater annexin V-Alexa 488 binding and chromatin condensation/fragmentation in γ-tocotrienol (γ-T3)-treated MDA-MB-231 cells. In representative photos A and B, cells were incubated for 4 hr with either 0 or 25 μg/mL γ-T3. The annexin V-Alexa 488-binding assay was then performed on the cells. Very few control cells (A) exhibited green fluorescence (reflecting annexin V-Alexa 488 binding to the cell surface), in contrast to γ-T3-treated cells (B). The number of cells positive for annexin V binding (per randomly selected, microscopic view) from four different experiments (mean ± SEM; $^*P < 0.01$ vs. control) is also shown (C). In representative photos D and E, cells were incubated for 72 hr with either 0 or 25 μg/mL γ-T3. The cells were then fixed and stained with the DNA-binding stain, DAPI. The staining revealed that control cells (D) had less condensed or fragmented chromatin than γ-T3-treated cells (E). More complete data from three different experiments (mean ± SEM; $^*P < 0.05$ vs. control) further show that more γ-T3-treated cells than control cells had condensed/fragmented chromatin (F).

3.3. Chromatin condensation and fragmentation in y-tocotrienol-treated cells

To further establish the apoptotic effect of γ-tocotrienol on MDA-MB-231 cells, DAPI staining was performed to detect the presence of condensed or fragmented chromatin, which is a cardinal late feature of apoptosis. As shown by the representative photos (Fig. 3D and E), condensed (smaller in size) and fragmented (irregularly shaped remnants) chromatin was more evident in cells after 72 hr of incubation with 25 μg/mL γ-tocotrienol. That is, this late apoptotic feature was present in a larger number of γ-tocotrienol-treated cells relative to the untreated cells, as revealed further by the bar graph data (Fig. 3F).

3.4. Change in MMP in y-tocotrienol-treated cells

Reduction and collapse of MMP that results in mitochondrial permeability transition (MPT) is among the very first intracellular events preceding the execution phase of apoptosis via the mitochondria-mediated death

pathway [8,10]. Moreover, MPT is often associated with the release of cytochrome c from mitochondria to cytosol. To determine if the MMP was reduced in γ-tocotrienol-treated

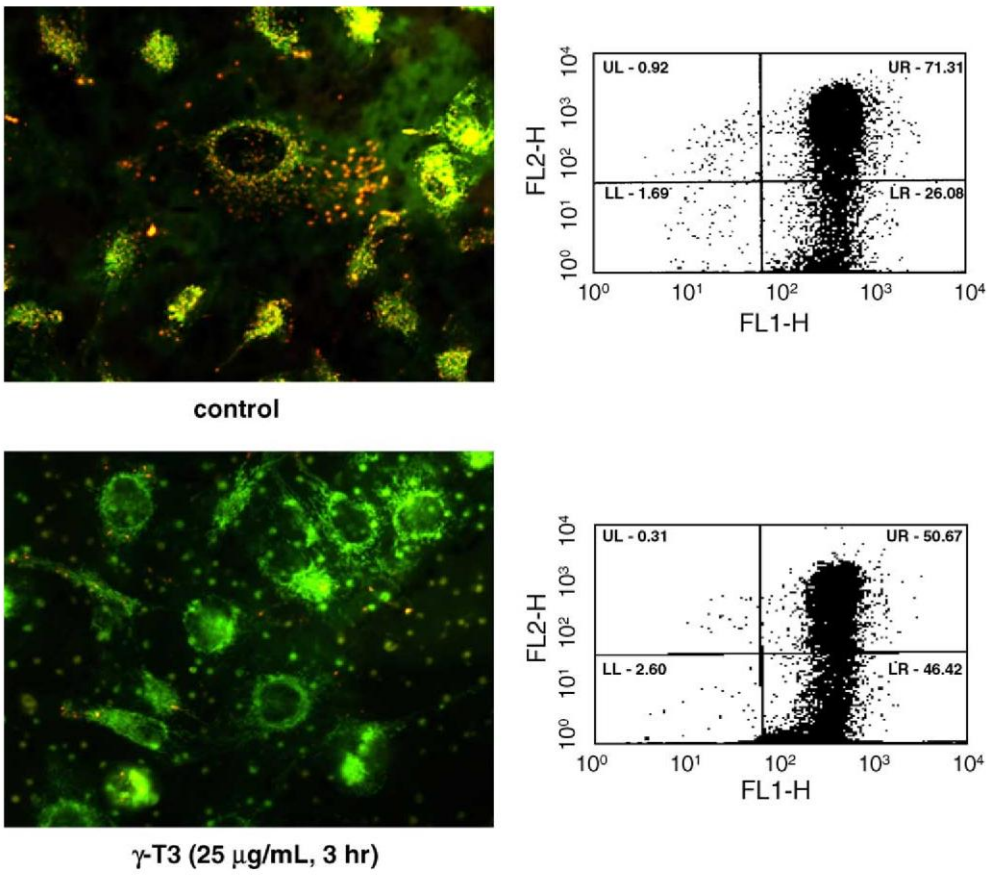

Fig. 4. Detection of mitochondrial membrane potential (MMP) reduction in γ-tocotrienol (γ-T3)-treated MDA-MB-231 cells. The cells were incubated for 3 hr with either 0 or 25 μg/mL γ-T3. The culture media were removed, and the fluorescence detection probe, DePsipher™, added on top of the cells for 15 min of incubation. The representative photos show aggregation of DePsipher™ within mitochondria of most of the control cells, resulting in the orange-red fluorescence. Cells that had been treated with γ-T3 exhibited mainly green fluorescence due to inability to aggregate DePsipher™ within their mitochondria, indicating reduction and collapse of the MMP. The flow cytometric histograms confirmed that the MMP was reduced in more of the γ-T3-treated cells than control cells. The results are representative of three different experiments. See Section 3.4 for more details.

MDA-MB-231 cells, change in the MMP was assessed indirectly by detecting the aggregation of the fluorescence probe, DePsipher™, in the mitochondria (Fig. 4). Aggregation of DePsipher™ requires intact MMP, which causes the probe to fluoresce orange-red instead of green as would be the case when DePsipher™ fails to aggregate due to reduced MMP. As shown by the representative photos, the control cells showed more orange-red fluorescence, indicative of the aggregation of DePsipher™ in mitochondrial membrane made possible by intact MMP. In contrast, the γ-tocotrienol-treated cells showed mostly green fluorescence because they were unable to aggregate DePsipher™ due to reduction and collapse of MMP. Moreover, in the example shown, flow cytometry revealed a larger percentage of the control cell population (71%) than the γ-tocotrienol-treated cell population (51%) having orange-red fluorescence (compare UR quadrant values). Conversely, a larger percentage of the γ-tocotrienol-treated cell population (46%) than control cell population (26%) had green fluorescence (compare LR quadrant values). Overall, however, the mean UR quadrant value for control cells was 76:08 ± 5:03 vs. 49:50 ± 9:87 for γ-tocotrienol-treated cells, which was significantly different ($P < 0:05$). Moreover, the mean LR quadrant value for γ-tocotrienol-treated cells was 47:02 ± 10:34 vs. 21:04 ± 5:62 for control cells, which was significantly different ($P < 0:05$). Taken together, these data suggested that MPT appeared when MDA-MB-231 cells were treated with γ-tocotrienol, and that this would eventually result in the release of mitochondrial proteins.

3.5. Release of cytochrome c from mitochondria into cytosol in y-tocotrienol-treated cells

During the initiation phase of apoptosis via the mitochondria-mediated death pathway, cytochrome c of mitochondria is released into the cytosol as a key event [8,10]. To determine if this happened in γ-tocotrienol-treated MDA-MB-231 cells, cytochrome c protein levels in both the mitochondrial and cytosolic fractions were determined by Western immunoblotting analysis (Fig. 5A). As can be seen, cytochrome c in the mitochondrial fraction seemed to start diminishing after just 3 hr, although cytochrome c was not able to be detectable in the cytosolic fraction until the

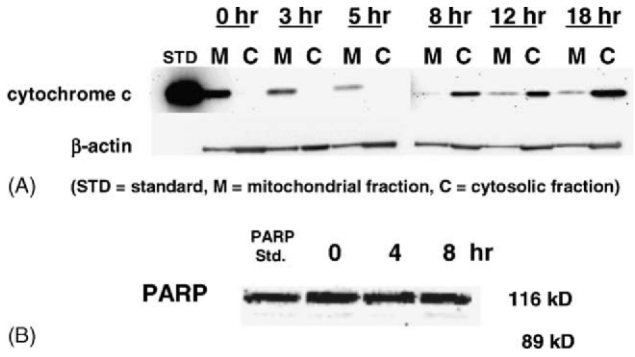

Fig. 5. Release of cytochrome *c* from mitochondria into cytosol (A) and absence of PARP cleavage (B) in MDA-MB-231 cells after treatmen with γ-tocotrienol. In panel A, the cells were incubated with 25 μg/mL γ-tocotrienol for 0–18 hr and the presence of cytochrome *c* in the mitochondrial and cytosolic fractions was determined by Western immunoblotting analysis. In panel B, cells were incubated with 25 μg/mL γ-tocotrieno for 4 and 8 hr, and Western immunoblotting analysis was performed to detect possible proteolytic modification of PARP (116 kD) into a fragmented protein product (89 kD) during the early stages of apoptosis. The results are representative of three different experiments.

8-hr time point. Nevertheless, these results suggest that cytochrome c was released from mitochondria into the cytosol as an early step in the mitochondria-mediated death pathway, which triggered the γ-tocotrienol-mediated induction of apoptosis.

3.6. Effects of g-tocotrienol on PARP, Bax, and Bcl-2

Released cytochrome c has a role in activating downstream caspases, such as caspase-3 [8,10]. Proteolytic cleavage of PARP (another early marker of apoptosis) is an indication that caspase-3 has been activated since caspase-3 cleaves PARP [14]. Change in the Bax:Bcl-2 ratio is known to regulate the appearance of MPT as well as release of cytochrome c from mitochondria [10]. Thus, it was logical to next determine if PARP cleavage and change in Bax/Bcl-2 expression occurred in γ-tocotrienol-treated MDA-MB-231 cells. As shown in Fig. 5B, intact PARP protein (standard) appeared as a 116 kD band. An 89 kD band, which is known to be the major proteolytic fragment produced from PARP cleavage by caspase-3, was not detectable after cells were treated for either 4 or 8 hr with 25 μg/mL γ-tocotrienol. Moreover, we were unable to find differences at 12, 18, and 24 hr (data not shown).

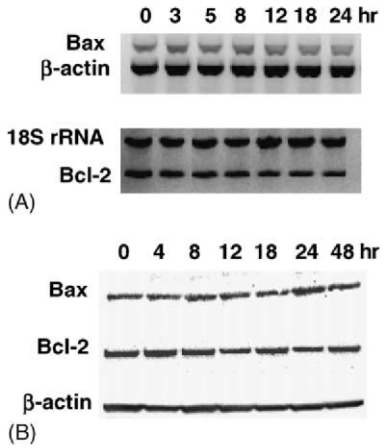

Fig. 6. No effect of γ-tocotrienol on the expression of Bax and Bcl-2 (mRNA and protein) in MDA-MB-231 cells. The cells were incubated with 25 μg/mL γ-tocotrienol for 0–48 hr. Expression of mRNA levels was determined by relative RT–PCR (A) using gene-specific primers and internal standard primer sets (β-actin for Bax, 18S rRNA for Bcl-2). Expression of protein levels was determined by Western immunoblotting analysis (B) using β-actin as the housekeeping protein. The results are representative of three different experiments.

As can be seen, the expression of Bax and Bcl-2 mRNA (Fig. 6A) and protein (Fig. 6B) did not change in MDAMB-231 cells after being incubated with 25 μg/mL ytocotrienol for broad periods of time. After normalizing these results by densitometric analysis, no significant differences were found (data not shown).

4. DISCUSSION

It was established previously that tocotrienols, including γ-tocotrienol, inhibit the growth or proliferation of human breast cancer cells [4–6]. Subsequently, some evidence was provided that tocotrienols can inhibit proliferation of human breast cancer cells by inducing apoptosis [7]. Consistent with this subsequent study, it was confirmed in the present study that γ-tocotrienol decreased the viability of MDA-MB-231 cells, as determined by trypan blue dye exclusion analysis. Thus, the anti-proliferative effect of γ-tocotrienol on MDA-MB-231 cells can be explained largely by its ability to cause cell death likely by inducing apoptosis. Supporting this view, three different standard assays established more definitively that γ-tocotrienol induced apoptosis. First, upon microscopic examination, morphological signs of apoptosis, such as membrane blebbing and presence of apoptotic bodies, were observed in the γ-tocotrienol-treated MDA-MB-231 cell samples. Secondly, the annexin V-Alexa 488-binding assay detected translocation of membrane PS from the inner to outer region of the plasma membrane of the cells, which is an early event of apoptosis. Thirdly, DAPI staining detected chromatin condensation and fragmentation in nuclei, which is a later characteristic feature of cells undergoing apoptosis. Therefore, these current findings substantiate and expand the previous research regarding the possible apoptotic effect of tocotrienols [7], and thus, prompted examination of mitochondria for involvement in the apoptosis induced by γ-tocotrienol.

Although γ-tocotrienol has substantial antioxidant activity due to its phenolic structure [1,2], it is uncertain if this chemical property was directly responsible for enabling γ-tocotrienol to trigger the events leading to apoptosis in MDA-MB-231 cells. Yet, other phenolic phytochemicals with substantial antioxidant activity are also capable of inducing apoptosis in cancer cells [21]. Hence, despite the apparent requirement for reactive oxygen species in mediating apoptosis [22], phenolic phytochemicals by themselves can induce, rather than prevent, apoptosis in cancer cells. Evidently, phenolic phytochemicals have other properties that enable them to kill cancer cells. Paradoxically, some phenolic phytochemicals can promote the formation of reactive oxygen species in inducing apoptosis [23]. One recent study has suggested that the flavonoid, baicalin, acts as a prooxidant in allowing baicalin to induce apoptosis in Jurkat T-leukemia cells via disruption of mitochondria

[24]. Thus, it is possible that exposing MDA-MB-231 cells to γ-tocotrienol created an oxidative cellular environment that triggered the molecular events leading to mitochondrial disruption and apoptosis.

Mitochondria have a central role in apoptosis [25]. Other results in the present study are consistent with the belief that the apoptosis that was induced by γ-tocotrienol in MDA-MB-231 cells involved the mitochondria-mediated apoptotic pathway. The mitochondria of γ-tocotrienol-treated cells were disrupted. More specifically, there was reduction of the MMP as indicated by the observation that γ-tocotrienol-treated cells had less ability to aggregate the DePsipher™ fluorescence probe into mitochondrial membrane. Reduction and eventual collapse of the MMP is among the earliest steps during the initiation phase of apoptosis [8]. This step precedes the execution phase of apoptosis where cellular components are dismantled by caspases. Regarding how changes in MMP triggers apoptosis [26], reduction of MMP is thought to result from opening of an inner mitochondrial membrane channel, i.e. the so-called permeability transition pore. This gives rise to MPT [27]. Hence, the opening allows unequal distribution of ions and respiratory substrates in the inner membrane of mitochondria, with consequent collapse of MMP and arrest of ATP synthesis. Swelling of the mitochondrial matrix follows, leading finally to loss of integrity of mitochondrial outer membrane. In light of these known processes, it is conceivable that γ-tocotrienol somehow triggered the emergence of MPT, leading to eventual disruption of mitochondria in MDA-MB-231 cells.

The reduction of MMP, as detected indirectly with DePsipher™, in γ-tocotrienol-treated MDA-MB-231 cells suggested the occurrence of downstream events that are characteristic of the mitochondria-mediated death pathway. Several proteins are released from mitochondria into cytosol once the MPT is present. For example, cytochrome c is released, which has important consequences [8,10]. Along with Apaf-1 and (d)ATP, the released cytochrome c forms a complex called the apoptosome, which subsequently associates with procaspase-9 that then undergoes proteolytic processing. Hence, caspase-9 is formed that activates downstream caspases, including caspase-3, which is the main executioner of cells. Although not all of these steps were examined, the present study showed that cytochrome c was released from mitochondria into cytosol when MDA-MB-231 cells were incubated with γ-tocotrienol to induce apoptosis.

What allows cytochrome c release during apoptosis is unknown, although there are two hypotheses [25]. First, cytochrome c release happens after the outer mitochondrial membrane has been ruptured following collapse of the MMP. Secondly, cytochrome c release occurs when certain Bcl-2-related proteins establish channels in the mitochondrial membrane. Bcl-2 is an anti-apoptotic protein. Moving from the cytosol, it inserts into the outer mitochondrial membrane, thereby preventing cytochrome c release and apoptosis [28,29]. In contrast, Bax is a pro-apoptotic protein. It is known that translocation of Bax from cytosol to mitochondria can occur after there is a reduction in MMP [30]. Insertion of Bax into the outer mitochondrial membrane triggers the release of cytochrome c [31], which allows apoptosis to proceed. Therefore, an increased ratio of Bax:Bcl-2 is expected to be associated with apoptosis, based on other studies with human breast cancer cells where apoptosis was induced by certain phytochemicals [32,33]. To the contrary, neither Bcl-2 nor Bax mRNA and protein expression were changed in MDA-MB-231 cells treated with γ-tocotrienol to induce apoptosis. However, it is unknown whether upstream modulators of Bax, such as tBID (truncated BID) that promotes conformational changes of Bax to permit Bax to insert into the outer mitochondrial membrane [34], are also unaffected in γ-tocotrienol-treated cells. The translocation of Bax from cytosol to mitochondria was not assessed in the present study. In any event, the current data show that reduction of MMP was detected at an earlier time point (3 hr) than when the release of cytochrome c from mitochondria to cytosol was detected (8 hr) after apoptosis was induced in MDAMB-231 cells by γ-tocotrienol, suggesting that the MMP reduction allowed the cytochrome c release.

According to the traditional paradigm that has been described [10], after cytochrome c is released from mitochondria, this is followed by activation of caspases, such as caspase-3. As a result, numerous critical intracellular proteins are degraded by caspase-3 during the early stages of apoptosis. For example, PARP (116 kDa), an enzyme involved in DNA repair, can be cleaved by caspase-3 to produce an 89 kDa fragment. In the present study, however, noticeable induction of PARP cleavage was unable to be detected in MDA-MB-231

cells exposed to γ-tocotrienol. Thus, this particular finding does not support the possibility that activation of caspase-3 was involved in γ-tocotrienol-induced apoptosis of MDA-MB-231 cells. As such, the effect of γ-tocotrienol contrasts with that of some other phytochemicals. For instance, genistein has been reported to induce apoptosis in MDA-MB-231 cells that was accompanied by caspase-3 activation and PARP cleavage [35]. Additionally, β-sitosterol induced apoptosis in MDAMB-231 cells that was accompanied by activation of caspases 8 and 9, as well as caspase-3 [36]. It is unknown if caspases 3, 8, and 9 are activated in γ-tocotrienol-treated MDA-MB-231 cells because no direct enzymatic assays were performed. However, in certain conditions with MDA-MB-231 cells, activation of caspase-3 may not actually be a required event for apoptosis to occur [37]. It is known that not all inducers of apoptosis depend on caspase-3 activation in order to cause cell death [38]. Furthermore, induction of apoptosis in MDA-MB-231 cells is not always accompanied by activation of effector and initiator caspases [39]. Although caspase-3 activation is considered a pivotal event in many situations of apoptosis, it has been suggested that apoptosis can also occur in the absence of caspase-3. A study that used MCF-7 human breast cancer cells, which do not express functional caspase-3 [40], demonstrated that apoptosis induced by neocarzinostatin proceeds via sequential activation of caspases 9, 7, and 6 [41]. Thus, the activation of caspase-3 is not always absolutely necessary for apoptosis to occur.

Taken together, the results imply that cytochrome c is not the critical protein released from mitochondria that triggers γ-tocotrienol-induced apoptosis in MDA-MB-231 cells. In addition to cytochrome c, there is another protein called apoptosis-inducing factor (AIF), which is also released from mitochondria into cytosol during the early stages of apoptosis. AIF is known to directly induce apoptosis independently of caspase-3 [42], and once AIF is released, it can in turn promote cytochrome c release. Therefore, it is conceivable that the apoptosis in γ-tocotrienol-treated MDA-MB-231 cells was mediated directly by AIF, rather than through cytochrome c release and caspase-3 activation. Nonetheless, it remains unproven that cytochrome c is not involved in γ-tocotrienol-induced apoptosis in MDA-MB-231 cells.

In summary, γ-tocotrienol induced clear features of apoptosis in MDA-MB-231 cells. The apoptosis apparently involved initiation of the mitochondria-mediated death pathway, as suggested by the mitochondrial disruption in which reduction of MMP and release of cytochrome c were both detected. However, activation of caspases and modulation of Bax/Bcl-2 did not seem to be involved in the apoptosis because caspase-mediated PARP cleavage and changes in the expression of Bax/Bcl-2 mRNA and protein were not found.

REFERENCES

[1] Packer L, Weber SU, Rimbach G, Molecular aspects of a-tocotrienol antioxidant action and cell signaling, J Nutr 2001;131:369S–73S.
[2] Kamal-Eldin A, Appelqvist LA, The chemistry and antioxidant properties of tocopherols and tocotorienols, Lipids 1996;31:671–701.
[3] Theriault A, Chao J-T, Wang Q, Gapor A, Adeli K, Tocotrienol: a review of its therapeutic potential, Clin Biochem 1999;32:309–19.
[4] Nesaretnam K, Guthrie N, Chambers AF, Carroll KK, Effect of tocotrienols on the growth of a human breast cancer cell line in culture, Lipids 1995;30:1139–43.
[5] Guthrie N, Gapor A, Chambers AF, Carroll KK, Inhibition of proliferation of estrogen receptor-negative MDA-MB-435 and –positive MCF-7 human breast cancer cells by palm oil tocotrienols and tamoxifen, alone and in combination, J Nutr 1997;127:544S–8S.
[6] Nesaretnam K, Stephen R, Dils R, Darbre P, Tocotrienols inhibit the growth of human breast cancer cells irrespective of estrogen receptor status, Lipids 1998;33:461–9.
[7] Yu W, Simmons-Menchaca M, Gapor A, Sanders BG, Kline K, Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols, Nutr Cancer 1999;33:26–32.
[8] Wilson MR, Apoptosis: unmasking the executioner, Cell Death Differ 1998;5:646–52.
[9] Nagata S, Apoptosis by death factor, Cell 1997;88:355–65.
[10] Budihardjo I, Oliver H, Lutter M, Luo X, Wang X, Biochemical pathways of caspase activation during apoptosis, Annu Rev Cell Dev Biol 1999;15:269–90.

[11]King P, Goodbourn S, STAT1 is inactivated by a caspase, J Biol Chem 1998;273:8699–704.
[12] Ravi R, Bedi A, Fuchs EJ, Bedi A, CD95 (Fas)-induced caspasemediated proteolysis of NF-kappaB, Cancer Res 1998;58:882–6.
[13] Wang X, Zelenski NG, Yang J, Sakai J, Brown MS, Goldstein JL, Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis, EMBO J 1996;15:1012–20.
[14] Duriez PJ, Shah μM, Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death, Biochem Cell Biol 1997; 75:337–49.
[15] Fujita N, Tsuruo T, Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis, Biochem Biophys Res Commun 1998;246:484–8.
[16] Diederich L, Fotedar A, Fotedar R, Proteolytic cleavage of retinoblastoma protein upon DNA damage and fas-mediated apoptosis, Cell Biol Toxicol 1998;14:133–40.
[17] Zhang Y, Fujita N, Tsuruo T, Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis, Oncogene 1999;18:1131–8.
[18] Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S, A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD, Nature 1998;391:43–50.
[19] Bossy-Wetzel E, Green DR, Assays for cytochrome c release from mitochondria during apoptosis, Methods Enzymol 2000;322: 235–42.
[20] Zhang G, Gurtu V, Kain SR, Yan G, Early detection of apoptosis using a fluorescent conjugate of annexin V. Biotechniques 1997;23: 525–31.
[21] Kuo SM, Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells, Cancer Lett 1996;110: 41–8.
[22] Sakaguchi N, Inoue M, Ogihara Y. Reactive oxygen species and intracellular Ca2þ, common signals for apoptosis induced by gallic acid, Biochem Pharmacol 1998;55:1973–81.
[23] Sakagami H, Arakawa H, Maeda M, Satoh K, Kadofuku T, Fukuchi K, Gomi K, Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants, Anticancer Res 2001; 21:2633–41.
[24] Ueda S, Nakamura H, Masutani H, Sasada T, Takabayashi A, Yamaoka Y, Yodoi J, Baicalin induces apoptosis via mitochondrial pathway as prooxidant, Mol Immunol 2002;38:781–91.
[25] Desagher S, Martinou J-C, Mitochondria as the central control point of apoptosis, Trends Cell Biol 2000;10:369–77.
[26] Crompton M, The mitochondrial permeability transition pore and its role in cell death, Biochem J 1999;341:233–49.
[27] Zoratti M, Szabo´ I, The mitochondrial permeability transition, Biochim Biophys Acta 1995;1241:139–76.
[28] Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD, The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis, Science 1997;275:1132–6.
[29] Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X, Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, Science 1997;275: 1129–32.
[30] Smaili SS, Hsu YT, Sanders KM, Russell JT, Youle RJ, Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential, Cell Death Differ 2001;8:909–20.
[31] Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC, Bax directly induces release of cytochrome c from isolated mitochondria, Proc Natl Acad Sci USA 1998;95:4997– 5002.
[32] Hong C, Firestone GL, Bjeldanes LF, Bcl-2 family-mediated apoptotic effects of 3,30-diindolylmethane (DIM) in human breast cancer cells, Biochem Pharmacol 2002;63:1085–97.
[33] Vergote D, Cren-Olive C, Chopin V, Toillon RA, Rolando C, Hondermarck H, Le Bourhis X, (—)-Epigallocatechin (EGC) of green tea induces apoptosis of human breast cancer cells but not of their normal counterparts, Breast Cancer Res Treat 2002;76:195–201.
[34] Roucou X, Montessuit S, Antonsson B, Martinou JC, Bax oligomerization in mitochondrial membranes requires tBid (caspase-8- cleaved Bid) and a mitochondrial protein, Biochem J 2002;368: 915–21.
[35] Li Y, Upadhyay S, Bhuiyan M, Sarkar FH, Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein, Oncogene 1999; 18:3166–72.
[36] Awad AB, Roy R, Fink CS, Beta-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells, Oncol Rep 2003;10:497–500.

[37] Zhang M, Guo R, Zhai Y, Yang D, LIGHT sensitizes IFNgammamediated apoptosis of MDA-MB-231 breast cancer cells leading to down-regulation of anti-apoptosis Bcl-2 family members, Cancer Lett 2003;195:201–10.
[38] Herzig MC, Liang H, Johnson AE, Woynarowska B, Woynarowski JM, Irofulven induces apoptosis in breast cancer cells regardless of caspase-3 status, Breast Cancer Res Treat 2002;71:133–43.
[39] Bao JJ, Le XF, Wang RY, Yuan J, Wang L, Atkinson EN, LaPushin R, Andreeff M, Fang B, Yu Y, Bast Jr RC, Reexpression of the tumor suppressor gene ARHI induces apoptosis in ovarian and breast cancer cells through a caspase-independent calpain-dependent pathway, Cancer Res 2002;62:7264–72.
[40] Janicke RU, Sprengart ML, Wati MR, Porter AG, Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis, J Biol Chem 1998;273:9357–60.
[41] Liang Y, Yan C, Schor NF, Apoptosis in the absence of caspase 3, Oncogene 2001;20:6570–8.
[42] Cande C, Cecconi F, Dessen P, Kroemer G, Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci 2002;115:4727–34.