

# Significance of mitochondrial calcium and nitric oxide for apoptosis of human breast cancer cells induced by tamoxifen and etoposide

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**Abstract.** In the present study, we tested the significance of mitochondria for apoptosis upon exposure to tamoxifen and etoposide using two human breast cancer cell lines, MCF-7 and MDA-MB-231. We showed that both tamoxifen and etoposide induced apoptosis, increased intramitochondrial calcium and nitric oxide, and decreased mitochondrial transmembrane potential in both cell lines. Both drugs increased mitochondrial protein tyrosine nitration and caused release of cytochrome c from the mitochondria of both cell lines. This study suggests that tamoxifen and etoposide utilize a common mechanism to induce apoptosis in MCF-7 and MDA-MB-231 cells.

## Introduction

Breast cancer, the second most common cause of death in women, is caused in part by insufficient apoptosis (1). Cancer cells are resistant to apoptosis, and inadequate apoptosis promotes both the initial phase of carcinogenesis and the development of chemotherapy resistance. Mitochondria play a crucial role in apoptosis (2-5). During apoptosis, mitochondria produce high levels of reactive oxygen and nitrogen species, the mitochondrial transmembrane potential ( $\Delta\psi$ ) declines, and mitochondrial intermembrane protein cytochrome c is released. Many cancer chemotherapeutic agents induce apoptosis via a mechanism involving mitochondria, nitric oxide (NO), and peroxynitrite. For example, tamoxifen (TAM) which induces apoptosis via mitochondria-dependent pathways (6,7) increases peroxynitrite in isolated mitochondria (8). Etoposide (ETP), a widely used drug of a different drug

family, has also been shown to induce apoptosis by inducing cytochrome c release in isolated mitochondria and by causing cell death via mitochondria-dependent pathways (9). It has also been suggested that ETP increases mitochondrial calcium, releases cytochrome c from mitochondria and induces apoptosis in Jurkat cells (10). Staurosporine is another chemotherapeutic agent that induces apoptosis in human breast cancer cells (11,12). In PC12 cells, staurosporine-induced apoptosis is mediated by elevated  $\text{Ca}^{2+}$  and increased oxidative stress, and is prevented by peroxynitrite scavengers (13). Doxorubicin, another cancer chemotherapeutic agent, induces apoptosis via increased oxidative stress and decreased  $\Delta\psi$  (14,15).

Intramitochondrial calcium  $[\text{Ca}^{2+}]_m$  is critical for apoptosis (16,17). Elevated  $[\text{Ca}^{2+}]_m$  induces apoptosis in various cells (13,18,19). This form of apoptosis requires increased NO synthase (NOS) activity (18,19) and is prevented by lowering mitochondrial superoxide (4,20) or by scavenging peroxynitrite (13). Mitochondrial NOS produces NO in response to elevated  $[\text{Ca}^{2+}]_m$  (21,22). A considerable portion of mitochondrial NO reacts with superoxide and forms peroxynitrite (22,23) that releases cytochrome c from the mitochondria and induces mitochondrial apoptosis (8,23,24). The interplay between  $[\text{Ca}^{2+}]_m$ , mitochondrial NO, and mitochondrial peroxynitrite for apoptosis of many cells including brain, heart and kidney has been studied (22). However, the significance of  $[\text{Ca}^{2+}]_m$ , mitochondrial NO, and peroxynitrite for human breast cancer cells is not well known. We recently showed that in isolated mitochondria TAM increases  $[\text{Ca}^{2+}]_m$  which stimulates mitochondrial NOS, elevates mitochondrial peroxynitrite, and causes mitochondrial apoptosis (8), and we hypothesized that  $[\text{Ca}^{2+}]_m$ , mitochondrial NO and peroxynitrite may play key roles in apoptosis of breast cancer cells. In the present study the mechanism of apoptosis in MCF-7 and MDA-MB-231 cells induced by two drugs of different families was tested. We showed that both TAM and ETP increased  $[\text{Ca}^{2+}]_m$  and mitochondrial NO, caused a decrease in  $\Delta\psi$ , increased mitochondrial protein tyrosine nitration, caused cytochrome c release and induced apoptosis in MCF-7 and MDA-MB-231 cells. Our findings suggest that TAM and ETP utilize a common mechanism to induce apoptosis in human breast cancer cells.

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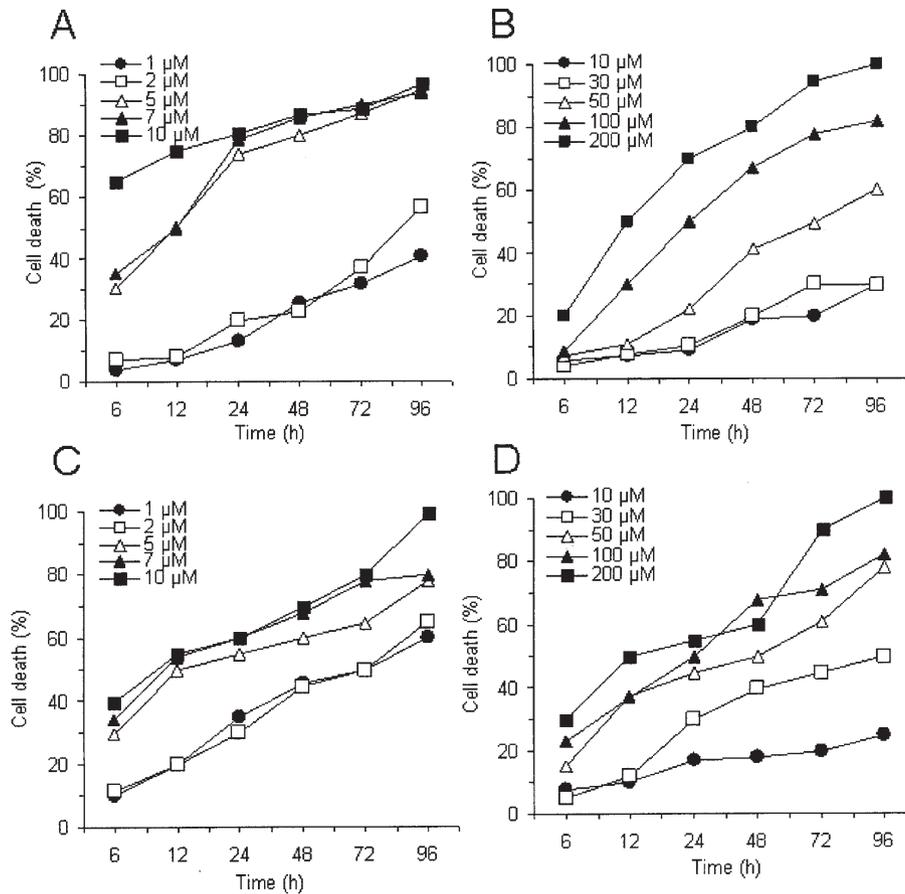


Figure 1. Dose- and time-response. (A) MCF-7 cells treated with varying doses of tamoxifen (TAM) showing  $LC_{50}$  of 5  $\mu$ M after 12 h of treatment. (B) MCF-7 cells treated with varying doses of etoposide (ETP) showing  $LC_{50}$  of 200  $\mu$ M after 12 h. (C) MDA-MB-231 cells treated with TAM showing  $LC_{50}$  after 12 h of treatment at 5  $\mu$ M. (D) MDA-MB-231 cells treated with ETP showing  $LC_{50}$  of 200  $\mu$ M after 12 h.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), rhodamine-2, AM (Rhod-2), tetramethylrhodamine methyl ester (TMRM), Hoechst 33342, mitotracker red, mitotracker green, and Alexa Fluor 488 were purchased from Invitrogen. 4,5-Diaminofluorescein diacetate (DAF) was purchased from Calbiochem. Monoclonal anti-nitrotyrosine antibody was purchased from Alexis. Monoclonal anti-cytochrome c antibody was purchased from eBioscience. Vectashield was purchased from Vector Laboratories. All other chemicals were purchased from Sigma.

**Cell culture.** MCF-7 and MDA-MB-231, two human breast cancer cell lines, were purchased from ATCC (Manassas, VA) and maintained in DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml) in a humidified incubator with 5%  $CO_2$  and 95% air at 37°C. Cells were seeded at a density of  $5 \times 10^5$ /ml in 24-well plates on 12-mm glass coverslips.

**Treatments.** For apoptosis induction, cells were cultured in DMEM containing FBS (5%). Cells were treated with TAM (1-10  $\mu$ M) or ETP (10-200  $\mu$ M) for 96 h. Control cells were treated with the same amount of vehicle.

**Hoechst staining assay.** Chromatin condensation was detected by staining with Hoechst 33342. Cells were grown on glass coverslips as described above. At 80% confluence, cells were treated with TAM and ETP. These cells were incubated with chromatin-specific Hoechst 33342 (3  $\mu$ g/ml) for 30 min to distinguish non-apoptotic cells from cells with apoptotic nuclear morphology (25). The fluorescent images of the control, TAM- and ETP-treated cells were captured at 750 nm by a Zeiss confocal laser scanning microscope (LSM 510) equipped with a multiphoton laser using a x20 objective. Cells with apoptotic nuclear morphology were scored.

**Intramitochondrial calcium.**  $[Ca^{2+}]_m$  was measured by loading cells with Rhod-2 (5  $\mu$ M) (26) and simultaneously with mitotracker green (200 nM). Initially, cells were loaded with Rhod-2 in DMEM and incubated at room temperature for 15 min followed by the addition of mitotracker green and further incubation for an additional 30 min under 5%  $CO_2$  at 37°C. After loading with Rhod-2 and mitotracker green, cells were permeabilized with digitonin (10  $\mu$ M) and washed with calcium-free Hank's balanced salt solution to eliminate the cytosolic fraction of Rhod-2 (27).

**Mitochondrial nitric oxide.** Mitochondrial NO was measured by using a membrane-permeable fluorescent probe DAF

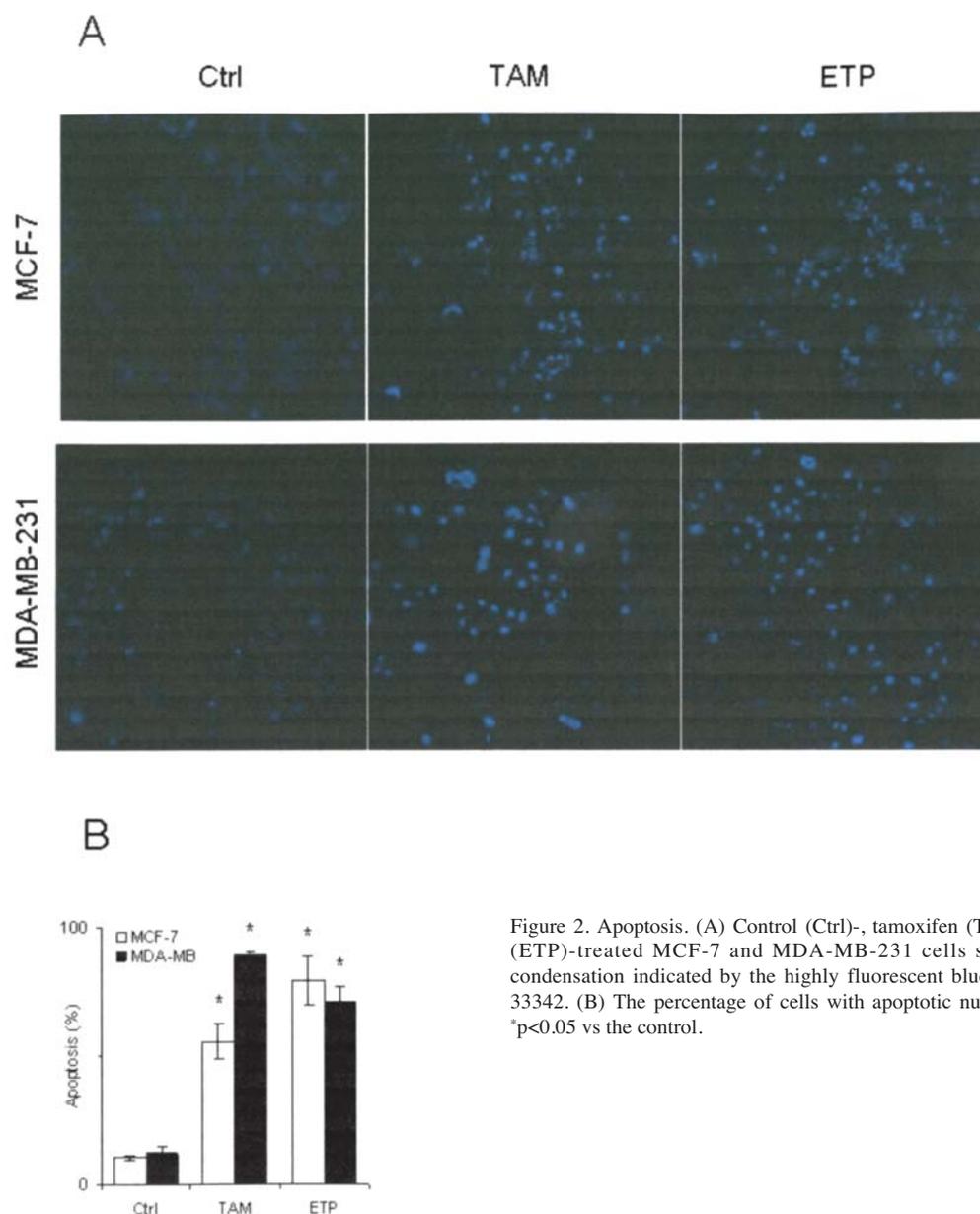


Figure 2. Apoptosis. (A) Control (Ctrl)-, tamoxifen (TAM)- and etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells showing chromatin condensation indicated by the highly fluorescent blue signals of Hoechst 33342. (B) The percentage of cells with apoptotic nuclei was determined. \* $p < 0.05$  vs the control.

(5  $\mu\text{M}$ ) (28). Initially, cells were incubated with mitotracker red (200 nM) for 5 min followed by incubation with DAF for an additional 15 min in DMEM under 5%  $\text{CO}_2$  at 37°C.

**Mitochondrial transmembrane potential.** The  $\Delta\psi$  was measured by loading cells with TMRM (10 nM) and mitotracker green (200 nM) in DMEM and by incubating in 5%  $\text{CO}_2$  at 37°C for 20 min (29).

**Protein tyrosine nitration and cytochrome c detection.** Cells were labeled with mitotracker red (500 nM) under 5%  $\text{CO}_2$  at 37°C for 45 min and fixed in paraformaldehyde (4%) for 15-20 min at room temperature. Cells were permeabilized with Triton X-100 (0.2%) followed by blocking in goat serum (10%). Cells were incubated with anti-nitrotyrosine primary antibody (1:250) or anti-cytochrome c primary antibody (1:200) overnight at 4°C, followed by incubation with Alexa Fluor 488 secondary antibody (1:200) for 1 h at room temperature and air dried. The cells were mounted with Vectashield on a glass slide.

**Fluorescent imaging.** Coverslips containing live cells were washed twice with a low fluorescence medium and mounted in a microchamber. Acquisition of fluorescence was performed in DMEM without phenol red, and images were obtained using a Zeiss confocal laser scanning microscope (LSM 510) using a x63 water objective. Fluorescence imaging of live cells loaded with different fluorescent dyes or fixed immunostained cells was performed using multichannel detection of excitation with the 488 nm line of an argon laser and the 543 and 633 lines of Helium Neon 1 and Helium Neon 2 and the 750 nm line of multiphoton laser at room temperature. Fluorescence images of cells loaded with mitotracker red, TMRM and Rhod-2 were acquired using 543-nm excitation and 579-nm emission. Fluorescence images of DAF, mitotracker green and Alexa Fluor 488 were acquired using 488-nm excitation and 516-nm emission. Images were acquired at 12-bit resolution by taking a single z-stack 1- $\mu\text{m}$  steps. Fluorescence images were analyzed using ImageJ software (National Institute of Health). Barograms are the mean  $\pm$  SEM of 6 images. Differences were tested by the

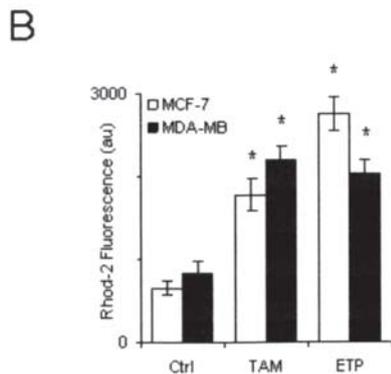
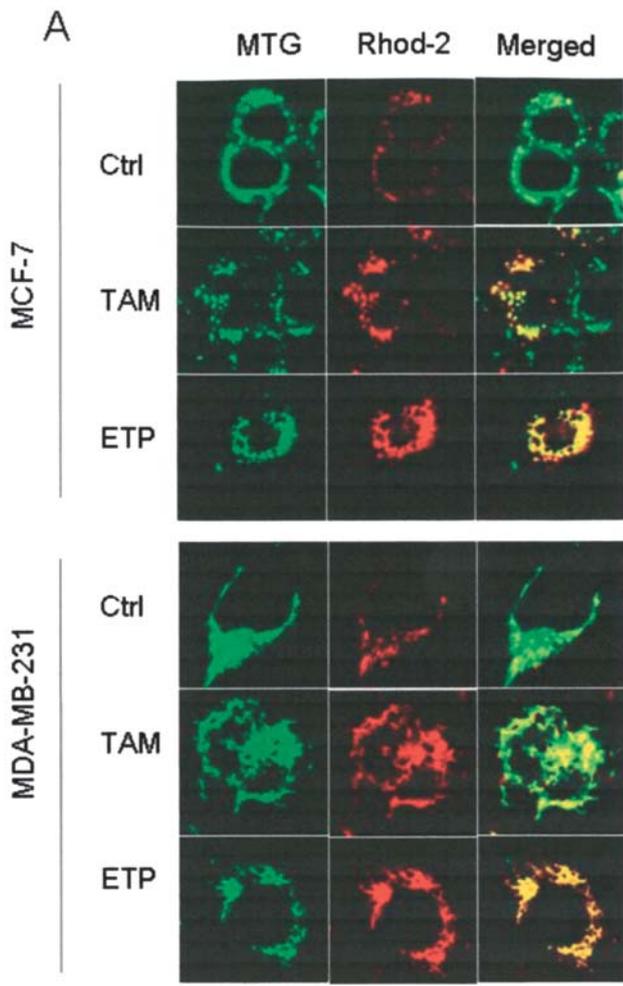


Figure 3. Intramitochondrial calcium  $[Ca^{2+}]_m$ . (A) Control (Ctrl)-, tamoxifen (TAM)- or etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells. Images of mitotracker green (MTG), Rhod-2, AM (Rhod-2) and merged are shown. Merged images are overlaid images of MTG and Rhod-2. (B)  $[Ca^{2+}]_m$  was measured after TAM or ETP treatment. \* $p < 0.05$  vs the control.

Student's t-test and considered statistically significant at  $p < 0.05$ .

**Results**

*Dose- and time-response.* Fig. 1A-D shows dose- and time-response curves for both cell lines treated with TAM or ETP.  $LC_{50}$  at 12 h of treatment was found to be 5  $\mu$ M for TAM and 200  $\mu$ M for ETP for both cell lines.

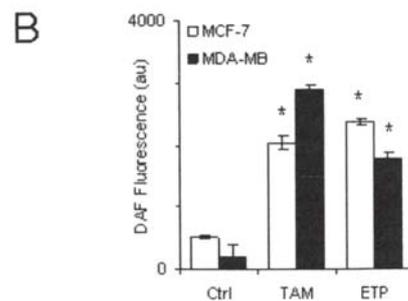
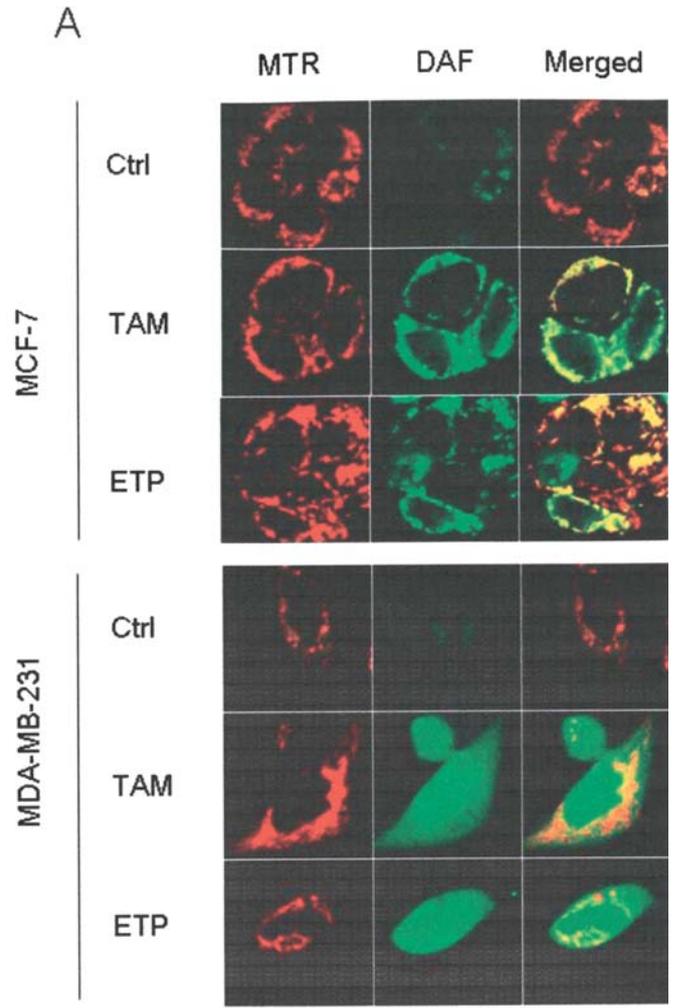


Figure 4. Intramitochondrial nitric oxide (NO). (A) Control (Ctrl)-, tamoxifen (TAM)- or etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells. Images of mitotracker red (MTR), DAF-2DA (DAF) and merged are shown. Merged images are overlaid images of MTR and DAF. (B) NO was measured after TAM or ETP treatment. \* $p < 0.05$  vs the control.

*TAM and ETP induce apoptosis.* Fig. 2A shows Hoechst 33342 fluorescence in MCF-7 and MDA-MB-231 cells. Apoptosis indicated by condensed nuclei with the intense blue fluorescence of Hoechst 33342 was increased by TAM and ETP in MCF-7 and MDA-MB-231 cells. Fig. 2B shows the increase in the percentage of apoptotic cells by TAM and ETP treatments in those cell types.

*TAM and ETP increase intramitochondrial calcium.* Fig. 3A and B shows an increase in the Rhod-2 fluorescence signal

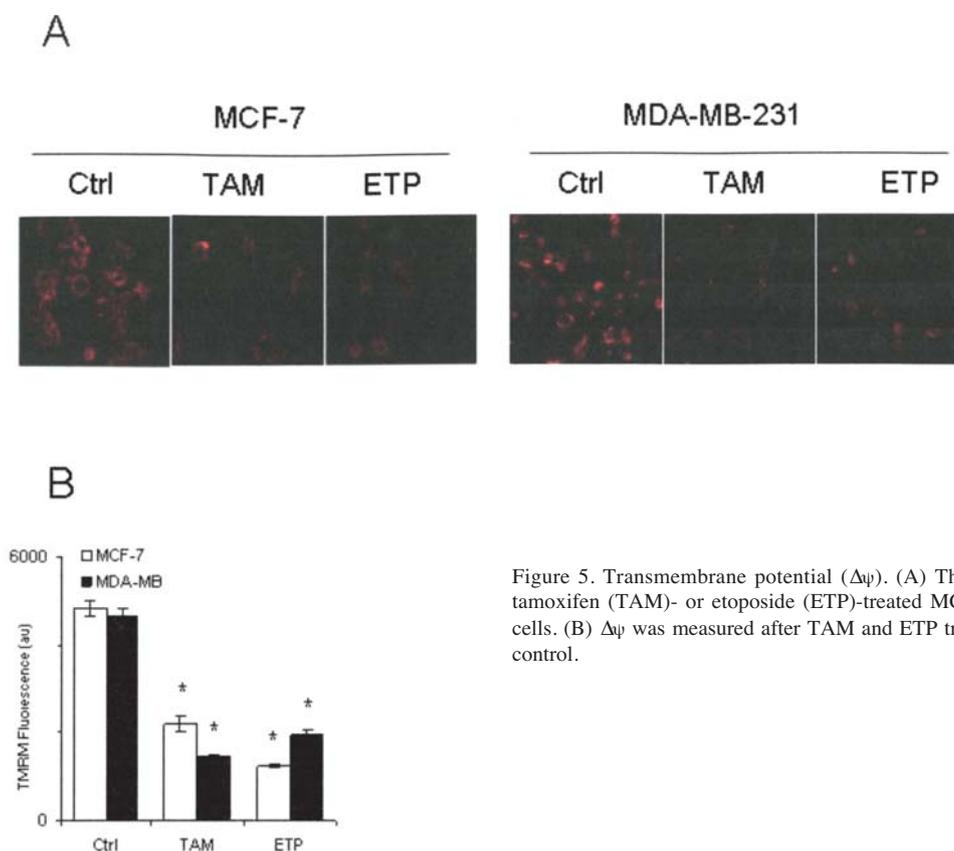


Figure 5. Transmembrane potential ( $\Delta\psi$ ). (A) The  $\Delta\psi$  of control (Ctrl)-, tamoxifen (TAM)- or etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells. (B)  $\Delta\psi$  was measured after TAM and ETP treatments. \* $p < 0.05$  vs the control.

after TAM and ETP treatments that co-localized within mitochondria in both cell types. These findings indicate that TAM and ETP increased  $[Ca^{2+}]_m$  in both MCF-7 and MDA-MB-231 cells. Barograms show the fluorescence intensity measurement of Rhod-2 indicating a significant increase in  $[Ca^{2+}]_m$  in comparison to the control.

**TAM and ETP increase mitochondrial nitric oxide.** Fig. 4A and B shows the increase in the DAF signal in TAM- and ETP-treated MCF-7 and MDA-MB-231 cells that were co-localized with mitotracker red (yellow fluorescence in merged images). Barograms show the fluorescence intensity measurement of the DAF signal in both cell lines treated with TAM and ETP. As shown, the fluorescence intensity was significantly higher in the mitochondria of TAM- and ETP-treated cells compared to the control cells, indicating increased NO in the mitochondria.

**TAM and ETP decrease mitochondrial transmembrane potential.** Decrease in TMRM fluorescence indicated decreased  $\Delta\psi$ . Fig. 5A and B shows a decrease in  $\Delta\psi$  in TAM- and ETP-treated MCF-7 and MDA-MB-231 cells. The bar diagram shows that the fluorescence intensity of the TMRM signal in MCF-7 and MDA-MB-231 cells treated with TAM and ETP were decreased indicating a decrease in  $\Delta\psi$ .

**TAM and ETP increase mitochondrial protein tyrosine nitration and induce cytochrome c release.** Fig. 6A and B shows a dramatic increase in mitochondrial protein tyrosine nitration immunoreactivity in TAM- and ETP-treated cells. The bar diagram shows a significant increase in tyrosine

nitration immunofluorescence intensity indicating a significant increase in nitrated tyrosine after TAM and ETP treatments. Fig. 7 shows the immunofluorescence intensity of cytochrome c that is punctated and co-localized within the mitochondria of control cells indicating localization of cytochrome c in the mitochondria, whereas in TAM- and ETP-treated cells cytochrome c is scattered in the cytoplasm, indicating the release of cytochrome c from the mitochondria.

## Discussion

The present study demonstrated that TAM and ETP induce apoptosis in both MCF-7 and MDA-MB-231 cells. Both drugs increased  $[Ca^{2+}]_m$  and mitochondrial NO, decreased  $\Delta\psi$ , increased mitochondrial protein tyrosine nitration, and caused cytochrome c release.

We first tested various concentrations of TAM and ETP to induce cell death in both cell lines. Fig. 1A-D shows the time course of various concentrations of TAM and ETP in both cells. As shown, 12 h of incubation of both cell lines with 5  $\mu$ M TAM or 200  $\mu$ M ETP caused 50% cell death. In order to confirm that the apoptotic cell death caused by TAM or ETP was apoptotic, the Hoechst assay was used. As shown in Fig. 1A-D, 5  $\mu$ M TAM or 200  $\mu$ M ETP caused apoptosis in both cell types. Therefore, these concentrations of TAM and ETP were used throughout the study.

Perturbed cellular  $Ca^{2+}$  homeostasis causes apoptosis (30-32).  $Ca^{2+}$ -dependent mitochondrial dysfunction is a critical event that leads to the release of cytochrome c from mitochondria (33). Fig. 3A and B shows that TAM and ETP caused a significant increase in  $[Ca^{2+}]_m$  in both types of human breast cancer cells. Mitochondria rapidly and efficiently store

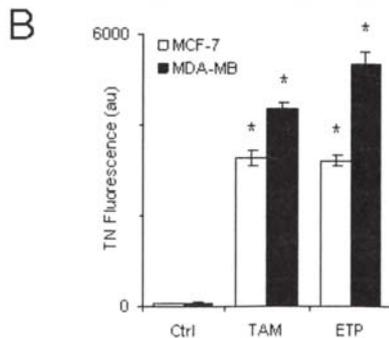
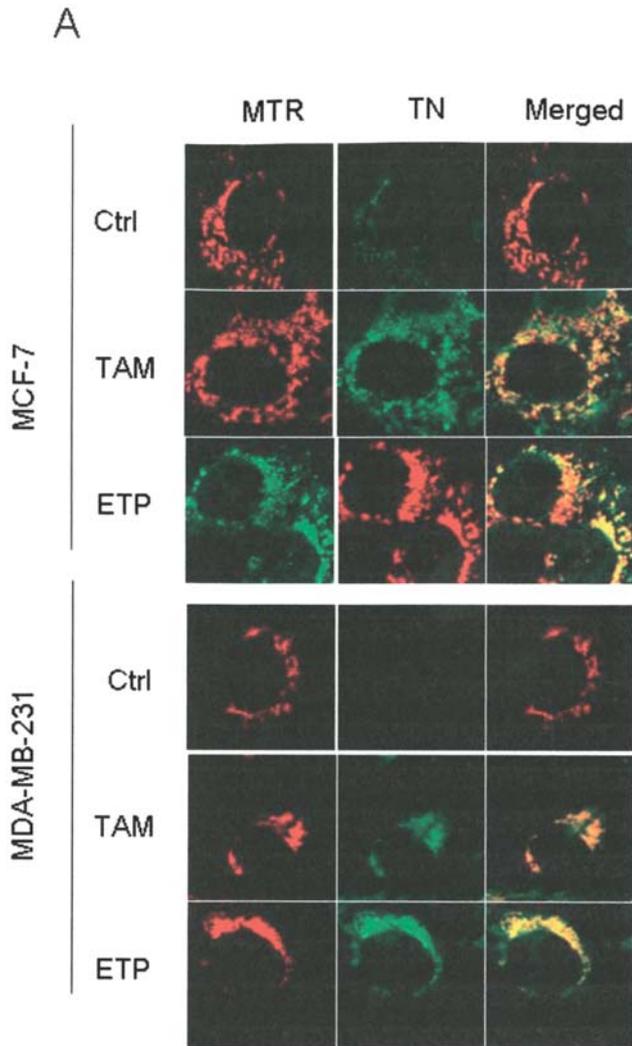


Figure 6. Protein tyrosine nitration (TN). (A) TN in the mitochondria of control (Ctrl)-, tamoxifen (TAM)- or etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells. Merged images show the overlay of mitotracker red (MTR) with anti-nitrotyrosine antibody immunoreactivity (TN). Higher co-localization in the mitochondria shows higher TN in the mitochondria. (B) Quantification of protein tyrosine nitration in the mitochondria of control (Ctrl)-, tamoxifen (TAM)- and etoposide (ETP)-treated cells. \* $p < 0.01$  vs the control.

large amounts of  $[Ca^{2+}]_m$ , however, mitochondria maintain very low  $[Ca^{2+}]_m$  by precipitating  $[Ca^{2+}]_m$  to matrix electron-dense granules (8,24,34-38). By precipitating and releasing  $[Ca^{2+}]_m$  from these granules, mitochondria maintain dynamic intra-organelle calcium homeostasis (8,24,39-42). Intramitochondrial calcium homeostasis can be altered by drugs,

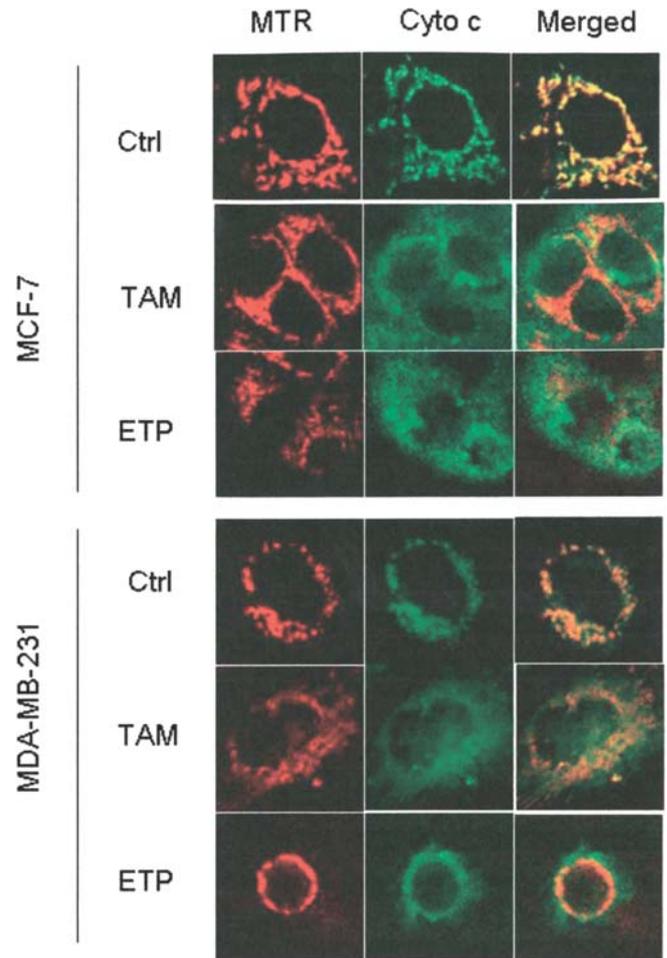


Figure 7. Cytochrome c (Cyto c) release. Cyto c in control (Ctrl)-, tamoxifen (TAM)- or etoposide (ETP)-treated MCF-7 and MDA-MB-231 cells. Merged images are of mitotracker red (MTR) with anti-cytochrome c reactivity (cyto c). Punctated cytochrome c signal in control cells shows localization of cyto c in the mitochondria. Diffused cyto c signal in TAM- and ETP-treated cells shows cyto c release from mitochondria.

hormones, or pathologic conditions (34-36,39-44). For example, vasopressin has been shown to decrease the matrix calcium granules in the mitochondria of neurons (40), and hypoxia increases the matrix granules in heart mitochondria (44). Recently, we showed that reperfusion after hypoxia increases  $[Ca^{2+}]_m$  in heart mitochondria stimulating mitochondrial NO formation (24). We showed that TAM increases  $[Ca^{2+}]_m$  in isolated rat liver mitochondria and in mitochondria isolated from MCF-7 cells, and suggested that elevated  $[Ca^{2+}]_m$  causes TAM-induced mitochondrial NOS stimulation (8). Fig. 3A and B shows that both drugs increased  $[Ca^{2+}]_m$  in both human breast cancer cells. These findings are consistent with our recent report on isolated mitochondria (8), and we propose that the elevation of  $[Ca^{2+}]_m$  could be considered a common response to TAM and ETP. Elevation of  $[Ca^{2+}]_m$  stimulates mitochondrial NOS activity which increases mitochondrial NO (8,23,24,45). Therefore, we tested mitochondrial NO upon treatment with TAM and ETP. As shown in Fig. 4A and B, TAM and ETP increased mitochondrial NO in both cell lines. Thus the present results confirm our previous findings on isolated mitochondria (8)



est that elevated mitochondrial NO might be in cell death caused by TAM and ETP.

NO decreases  $\Delta\psi$  via inhibition of cytochrome c oxidase (46,47). Decreased  $\Delta\psi$  is an early event in apoptosis which is followed by release of cytochrome c (48). Apoptosis involving decreased  $\Delta\psi$  and cytochrome c release has been demonstrated in MCF-7 cells after irradiation (49). As shown in Fig. 5A and B, TAM and ETP decreased  $\Delta\psi$  in both cancer cell lines. Other reports have also shown a decrease in  $\Delta\psi$  by anticancer chemotherapeutic drugs. For example, TAM decreased the  $\Delta\psi$  in MCF-7 cells (50) and in isolated mitochondria (8). Thus, the findings illustrated in Fig. 5A and B suggest that the decrease in  $\Delta\psi$  is associated with apoptosis induced by both TAM and ETP.

High levels of NO induce apoptosis in tumor cells via peroxynitrite formation (51). Peroxynitrite has a very short lifetime in most biological environments. Therefore, protein tyrosine nitration has been used as a peroxynitrite biomarker (8). Fig. 6A and B shows increased protein tyrosine nitration in cells treated with TAM and ETP. These data indicate that TAM and ETP treatment increased mitochondrial NO, leading to the generation of peroxynitrite. Previous findings indicate that the majority of tyrosine-nitrated protein in muscle cells are located in the mitochondria (52). The present study supports these reports and shows that upon treatment of cancer cells with TAM and ETP, a large amount of tyrosine nitration was found in the mitochondria suggesting that mitochondrial peroxynitrite might contribute to apoptosis induced by TAM and ETP.

Release of cytochrome c is a key event during apoptosis (2). Upon loss of  $\Delta\psi$  and release of cytochrome c, most cells are committed to apoptosis (53). Changes in mitochondrial function induced by different apoptotic stimuli are associated with release of cytochrome c into the cytosol (54,55). Peroxynitrite induces cytochrome c release by tyrosine nitration (56). Since TAM and ETP induced apoptosis (Fig. 2A and B) and caused elevation of mitochondrial peroxynitrite (Fig. 4A and B), we tested cytochrome c release in cells treated with TAM or ETP. Fig. 7 shows that in the control cells, cytochrome c co-localized within the mitochondria, whereas TAM- and ETP-treated cells showed diffused cytochrome c signals indicating redistribution of cytochrome c into the cytosol. Thus, apoptosis induced by TAM and ETP in both types of cells is associated with the release of cytochrome c.

We also demonstrated that changes in mitochondrial functions are critically associated with cell death caused by treatment with both TAM and ETP by a common mechanism. TAM and ETP are prototypes of two drug families with different known mechanisms of actions. TAM and ETP induced apoptosis in two human breast cancer cell lines MCF-7 and MDA-MB-231 by a very similar mechanism consisting of increased  $[Ca^{2+}]_m$  and mitochondrial NO, decreased  $\Delta\psi$ , as well as increased mitochondrial peroxynitrite and cytochrome c release from mitochondria. Breast cancer is a multifactor disease and standardized treatments are not available for patients. An understanding of the mechanisms underlying cell death by TAM and ETP may be helpful in developing new therapeutic agents and in improving the selectivity of the treatment.

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