Methylseleninic acid synergizes with tamoxifen to induce caspase-mediated apoptosis in breast cancer cells

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Abstract
Tamoxifen has efficacy as a breast cancer therapy and chemoprevention agent. However, toxicity and resistance to tamoxifen limit its clinical application. There is an urgent need to develop compounds that may be combined with tamoxifen to improve efficacy and overcome toxicity and resistance. We showed previously that the organoselenium compound methylseleninic acid (MSA) increased the growth-inhibitory effect of tamoxifen and reversed tamoxifen resistance in breast cancer cells. In this study, we examined the mechanism for induction of apoptosis by MSA combined with tamoxifen in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. 4-hydroxytamoxifen (TAM; 10⁻⁷ mol/L) alone resulted in cell cycle arrest but no apoptosis, whereas MSA alone (10 μmol/L) induced apoptosis in tamoxifen-sensitive cells. Combination of MSA with TAM resulted in a synergistic apoptosis in both tamoxifen-sensitive and tamoxifen-resistant breast cancer cells compared with either agent alone. MSA and TAM combined with TAM induced apoptosis through the intrinsic, mitochondrial apoptotic pathway. MSA induced a sequential activation of caspase-9 and then caspase-8. These results indicate that the growth inhibition synergy and reversal of tamoxifen resistance by combination of selenium with tamoxifen occurs via a tamoxifen-induced cell cycle arrest, allowing more cells to enter the intrinsic apoptotic pathway elicited by selenium. [Mol Cancer Ther 2008;7(9):3056–63]

Introduction
As a first-line nonsteroidal antiestrogen drug, tamoxifen has been widely prescribed as hormonal adjuvant therapy for early and advanced breast cancer in premenopausal and postmenopausal women and as a chemopreventive (1, 2). Tamoxifen binds to estrogen receptor α (ERα) and antagonizes estrogen-mediated growth of breast cancers. Despite the benefits of tamoxifen, 50% of breast cancer patients with ERα-positive tumors do not respond to tamoxifen therapy and almost all tamoxifen-responsive patients develop resistance to therapy (3–5). ER signaling remains functional in the majority of breast cancers that exhibit tamoxifen resistance and these resistant tumors are still dependent on ER for growth. This highlights an urgent need to develop new and non-cross-resistant therapeutic strategies designed to delete ERα protein and/or disrupt ER signaling by mechanisms that are distinct from that for tamoxifen.

Organic selenium compounds are highly effective chemopreventive agents with well-documented benefits in reducing total cancer incidence and mortality rates for several cancers (6–15). Organic selenium compounds have fewer side effects and lack the genotoxic action of inorganic selenium compounds such as selenite (6, 16, 17). Methylseleninic acid (MSA), a rapidly metabolized organoselenium compound useful in cell cultures studies, also does not exhibit toxic effects on cultured cells (18).

Although clinical trials with selenium are currently limited to chemoprevention, recent studies have explored the possibility of using selenium as a therapeutic for overt cancer through combination regimens with well-established chemotherapeutic and hormonal therapy drugs. Synergistic tumour growth inhibition and reduced toxicity were shown by combination of organic selenium with the topoisomerase I poison irinotecan or doxorubicin in tumour-bearing mice inoculated with cancer cells sensitive and resistant to irinotecan (19, 20). Similar reports of synergy between selenium and chemotherapeutic drugs were recently reported in breast cancer (20).

Previous studies in prostate cancer have shown that selenium induces apoptosis through caspase-8 activation and its cross-talk with multiple caspases (21). In MCF-7 breast cancer cells, Li et al. (20) showed that selenium combined with the cancer chemotherapeutic agent doxorubicin resulted in enhanced apoptosis. The combination induced apoptosis through endoplasmic reticulum stress followed by a direct release of caspases from the endoplasmic reticulum as well as an indirect activation of both intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways. These findings indicate that synergistic cell killing by selenium combined with apoptosis-inducing chemotherapeutic agents involved complicated mechanisms. The mechanisms by which tamoxifen synergizes with selenium are likely different given that therapeutic levels of tamoxifen result in cytostasis without significant apoptosis (22, 23). In this study, we sought to
discern the mechanisms of apoptosis induction by selenium combined with tamoxifen in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells through detailed analysis of apoptotic pathways.

Materials and Methods

Chemicals and Reagents

MSA (CH$_2$SeO$_2$H) was obtained from PharmaSe. 4-Hydroxytamoxifen (TAM) was obtained from Sigma. G$_1$ cell cycle blocking agent aphidicolin (APC) from Nigrospora sphaerica was purchased from Sigma. The irreversible caspase inhibitors Z-VDAD-FMK (general caspase inhibitor), Z-IEETD-FMK (caspase-8 inhibitor), and Z-LEHD-FMK (caspase-9 inhibitor) were obtained from R&D Systems. Propidium iodide was purchased from Molecular Probes. The Cell Death ELISA kit was purchased from Roche. All other reagents were purchased from Sigma-Aldrich and Fisher Scientific.

Cell Culture

MCF-7 cells (tamoxifen-sensitive, ER-positive human breast cancer cell line) were purchased from the American Type Culture Collection. MCF-7-LCC2 cells (tamoxifen-resistant, ER-positive breast cancer cell line) were kindly provided by Robert Clark (24). MCF-7 and MCF-7-LCC2 cells were cultured as described previously (25).

Apoptosis ELISA Assay

The induction of apoptosis by selenium was assayed using the nucleosome ELISA kit (Roche). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) after apoptotic cell death. Breast cancer cells were incubated with vehicle, APC, TAM (10$^{-7}$ mol/L), and/or MSA (10 $\mu$mol/L) for 24, 36, and 48 h in 96-well plates. The induction of apoptosis was evaluated by assessing the enrichment of nucleosomes in cytoplasm per manufacturer’s protocol.

Immunoblotting

The cells were collected, washed with cold PBS, and resuspended in high-salt lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 0.4 mol/L NaCl, 1 mol/L EDTA, 1 mol/L EGTA, 10 mmol/L β-mercaptoethanol, 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 1:100 Halt Protease Inhibitor Cocktail (Pierce Biotechnology)]. The lysates were placed on ice for 10 min, vortexed for 15 s, subjected to one freeze/thaw, and centrifuged at 20,000 x g at 4°C. Laemmli sample buffer was added to the supernatant the samples boiled 5 min, resolved by SDS-PAGE on a 12% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were incubated with antibodies against poly(ADP-ribose) polymerase (PARP), Bim, cytochrome c, caspase-2, -7, -8, -9, -10, and -12 (1:1,000; Cell Signaling Technology), cleaved caspase-8 (1:500), and tubulin (1:1,000; Santa Cruz Biotechnology). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology) or Fluor-conjugated secondary antides (1:5,000; Li-Cor Biosciences) and visualized by enhanced chemiluminescence or Odyssey fluorescence scanner (Li-Cor Biosciences). Densitometric analysis was done with the ImageJ software.3

Cell Cycle Analysis

To determine cell cycle distribution, 3 x 10$^5$ cells were plated in six-well plates and incubated with TAM (10$^{-7}$ mol/L) and/or MSA (10 $\mu$mol/L) for 24, 36, and 48 h. After treatment, the cells were collected by trypsinization and then fixed in 70% cold ethanol. Cells were washed with PBS and resuspended in 0.5 mL PBS containing 1 mg/mL RNase and 50 μg/mL propidium iodide. Following incubation in the dark for 30 min at room temperature, the cells were analyzed with flow cytometer (BD LSRII analyzer; BD Biosciences). The Multicycle software (Phoenix Flow Systems) was used to evaluate the data.

Statistical Analysis

Data were expressed as mean ± SD. Statistical comparisons of the results were made using t test and χ² test.

Results

Selenium + Tamoxifen Result in Synergistic Apoptosis in MCF-7 and MCF-7-LCC2 Cells

We showed previously that MSA combined with TAM resulted in synergistic growth inhibition in ERα-positive breast cancer cells (25). In this article, we will use the term “synergy” to indicate that the apoptosis induced by combination of selenium + tamoxifen was statistically greater than the sum of apoptosis by each agent. To determine whether the synergy was a result of increased apoptosis, an ELISA-based DNA fragmentation assay was used in tamoxifen-sensitive MCF-7 cells and the tamoxifen-resistant MCF-7-LCC2 cells. MCF-7-LCC2 is a stable tamoxifen-resistant population that was derived from a stepwise in vitro selection of the hormone-independent human breast cancer variant MCF-7-LCC1 and still retain levels of ERα expression comparable with the parental MCF-7 cells. In contrast to MSA, TAM alone did not induce significant apoptosis in either cell line (Fig. 1A and B). Coincubation of MSA with TAM resulted in significantly more apoptosis in both MCF-7 and MCF-7-LCC2 cells (Fig. 1A and B). Statistical analysis confirmed a synergistic interaction of MSA + TAM for inducing apoptosis (P < 0.05). At higher doses of tamoxifen (5 x 10$^{-6}$ mol/L) that induced apoptosis in MCF-7 but not MCF-7-LCC2 cells, cotreatment with MSA resulted in comparable levels of apoptosis, suggesting that doses of tamoxifen that induce apoptosis do not significantly contribute to the overall apoptosis induced by MSA (Fig. 1C and D).

Selenium and Selenium + Tamoxifen Induce Apoptosis through the Mitochondria Pathway

To determine whether apoptosis occurred through the intrinsic or extrinsic apoptosis pathways, activation of

3 http://rsb.info.nih.gov/ij/
proteins in both pathways was measured by Western blotting in both MCF-7 and MCF-7-LCC2 cells. Proteins measured were cleaved caspase-1, -2, -7, -8, -9, -10, and -12, Bim, cytochrome c, and PARP (Fig. 2A and B). TAM alone had no effect on activation of apoptotic proteins. Following incubation with MSA or MSA + TAM, activation (cleavage) of caspase-7, -8, and -9, PARP, and Bim and release of cytochrome c were detected in both MCF-7 and MCF-7-LCC2 cells. There was a consistently higher level of basal and induced cytochrome c in MCF-7 compared with MCF-7-LCC2 cells. Activated caspase-1, -2, -10, and -12 was not detected with any treatment (data not shown). In both cell lines, combination of MSA with TAM yielded greater activation of Bim, caspase-7, and PARP and more cytochrome c release than MSA alone, which is accordant with the synergistic effect shown by the apoptosis ELISA assay (Fig. 1).

Tamoxifen and MSA Induce G1 Arrest

Although \(10^{-7}\) mol/L TAM did not induce apoptosis in MCF-7 or MCF-7-LCC2 cells, TAM treatment resulted in significant G1 arrest in both cell lines (Fig. 3A and B). In tamoxifen-sensitive MCF-7 cells, TAM caused accumulation of cells in G1 (81.6% TAM versus 54.5% vehicle), whereas MSA had no effect on G1 accumulation (Fig. 3A). Combination of MSA with TAM resulted in similar G1 accumulation (81.1%). In contrast to MCF-7 cells, in MCF-7-LCC2 cells, both TAM and MSA resulted in G1 accumulation compared with vehicle (62.3% TAM and 59.3% MSA versus 39.3% vehicle). The combination of both agents resulted in greater G1 accumulation (78.5%) than either agent alone. A sub-G1 peak that is often attributed to fragmented DNA from apoptotic cells was not detected by fluorescence-activated cell sorting analysis after incubation of cells with MSA or MSA + TAM. As would be expected, the Cell Death ELISA assay that detected apoptosis in Fig. 1 is a more sensitive apoptosis assay than fluorescence-activated cell sorting analysis. In addition, the results do not rule out the possibility that some cells may have entered apoptosis from the S or G2-M phase of the cell cycle or an aneuploid population may be undergoing apoptosis.

Figure 1. Selenium + TAM result in synergistic apoptosis in breast cancer cells. We have used the term “synergy” to indicate that the apoptosis induced by combination of selenium + tamoxifen was statistically greater than the sum of apoptosis by each agent. Apoptosis assay of MCF-7 (A) and MCF-7-LCC2 (B) cells after 36 h incubation with MSA (10 μmol/L), TAM (10^{-7} mol/L), or MSA + TAM. C and D, apoptosis assay of MCF-7 and MCF-7-LCC2 cells after 36 h incubation with MSA (10 μmol/L), titrated TAM (10^{-7} mol/L - 5 × 10^{-6} mol/L), or both. C, MCF-7 cells. *, \(P < 0.05\), versus lower concentration of TAM. D, MCF-7-LCC2 cells. *, \(P < 0.05\), versus MSA + TAM (10^{-7} mol/L). Representative of three or more identical experiments. Mean ± SE (n = 3) of specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm using the following formula: absorbance of the sample / absorbance of the negative control (vehicle). *, \(P < 0.05\), statistical evidence of a synergistic effect in the combination of MSA and TAM compared with the sum of single-agent effects.
To determine if MSA could stimulate apoptosis of G1-phase MCF-7 and MCF-7-LCC2 cells, the G1 cell cycle blocking agent APC was used to arrest cells in G1 before incubation of cells with MSA. MSA induced apoptosis in MCF-7 and MCF-7-LCC2 cells following APC treatment (Fig. 3C and D). These data indicate that G1-arrested breast cancer cells can undergo apoptosis on incubation with MSA.

**Inhibition of Caspase-8 and -9 Prevents Selenium-Induced Apoptosis in MCF-7 and MCF-7-LCC2 Cells**

Results from Fig. 2 implicate both intrinsic (caspase-9 cleavage) and extrinsic (caspase-8 cleavage) pathways in the apoptotic response to selenium and selenium + TAM. To investigate the contribution of caspase-8 and -9 to MSA-induced apoptosis, MCF-7 and MCF-7-LCC2 cells incubated with MSA or MSA + TAM were coincubated with a general caspase inhibitor or specific inhibitors of caspase-9 and -8. The general caspase inhibitor Z-VAD-FMK completely blocked apoptosis by MSA or MSA + TAM in both MCF-7 and MCF-7-LCC2 cells (Fig. 4A and B). Remarkably, specific inhibitors of either caspase-8 or -9 blocked apoptosis by MSA and MSA + TAM to the same degree as the pan-caspase inhibitor (Fig. 4). The complete, and not partial, blockage of apoptosis by each specific inhibitor alone suggested that activation of caspase-8 and -9 by MSA and MSA + TAM occurred in a linear pathway and may not be due to parallel activation of both intrinsic (caspase-9) and extrinsic (caspase-8) pathways. Although crosstalk between caspase-8 and -9 has been shown in other cancer cells (26–30), the present data suggested that caspase activation may be dependent on each other.

**Inhibition of Caspase-9 Blocks Activation of Caspase-8 by Selenium**

To determine whether selenium-induced activation of caspase-8 and -9 occurred in a linear fashion, the effect of caspase-8 and -9 inhibitors on MSA-induced cleavage of caspase-8 and -9 was measured. The caspase inhibitors used are specific peptide inhibitors that bind to activated caspases. The inhibitors themselves have no effect on the MSA-induced cleavage of the caspases but instead inhibit the enzymatic activity of the activated caspases. Therefore, if the enzymatic activity of one caspase is required for the activation (cleavage) of another caspase, then a peptide inhibitor of the first caspase would prevent cleavage of the second caspase. Incubation of MCF-7 and MCF-7-LCC2 cells with a caspase-9 inhibitor prevented MSA-induced cleavage of caspase-8 (Fig. 5A and B). However, incubation of either cell line with caspase-8 inhibitor did not prevent MSA-induced cleavage of caspase-9. These data suggest that the cleavage of caspase-8 induced by MSA required a prior activation of caspase-9.

**Discussion**

Our previous studies showed a novel combination therapy regimen of tamoxifen + selenium that improved tamoxifen efficacy against breast cancer cells in culture. This study has now further shown that part of the therapeutic synergy between selenium and tamoxifen occurs through increased apoptosis and reversal of tamoxifen resistance. TAM alone (10⁻⁷ mol/L) did not induce apoptosis in either tamoxifen-sensitive MCF-7 or tamoxifen-resistant MCF-7-LCC2 cell lines, whereas MSA (10 µmol/L) induced apoptosis in both cell lines. MSA + tamoxifen resulted in a significant enhancement of apoptosis. These data indicated that MSA + tamoxifen could synergize in both tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. The growth inhibition advantage of combining selenium with tamoxifen was due primarily to an augmented response in both cell cycle arrest and apoptosis. The data also showed that a direct and complete crosstalk from caspase-9 to caspase-8 was required for selenium-induced apoptosis as specific inhibitors of both caspases completely, and not partially, blocked apoptosis. These combinations may provide enhanced efficacy by several means including delay in onset of tamoxifen resistance through lower tamoxifen doses, reversing tamoxifen resistance, and ameliorating the toxic side effects of tamoxifen in the uterus.
Several studies have shown that MSA can modulate other cancer chemotherapeutic agents resulting in elevated apoptosis. MSA was shown to synergize with TRAIL in prostate cancer cells (31) and with doxorubicin in breast cancer cells (20). Our data also showed a strong interaction occurs between selenium and ERα signaling in breast cancer in vitro, suggesting the possibility that combination of selenium with tamoxifen may improve antitumor efficacy similar to the improvement shown with selenium and irinotecan or doxorubicin (19, 20). The studies combining selenium with chemotherapeutic agents used 5 μmol/L MSA, a concentration that did not enhance apoptosis alone but when combined with a subapoptotic dose of chemotherapeutic agents could induce significant apoptosis. In the present study, we found that 5 μmol/L MSA alone did not induce apoptosis and addition of tamoxifen to 5 μmol/L MSA did not increase apoptosis (data not shown). It was only when a higher concentration of MSA was used (10 μmol/L) that MSA + TAM resulted in synergistic apoptosis in both MCF-7 and MCF-7-LCC2 breast cancer cells. The major growth-inhibitory mechanism for tamoxifen in breast cancer is cell cycle inhibition, and not apoptosis (22, 23), which results with agents such as doxorubicin. These likely accounts for the higher selenium concentrations required for apoptosis synergy when selenium was combined with tamoxifen.

Figure 3. Cell cycle analysis of MCF-7 and MCF-7-LCC2 cells after incubation of cells with vehicle (Veh), 10⁻⁷ mol/L TAM, 10 μmol/L MSA, APC, and combinations for 24 h. A, in MCF-7 cells, 10⁻⁷ mol/L TAM induced G1 accumulation (81.6%) compared with vehicle (*, \( P < 0.05 \)), whereas MSA had no significant effect. B, in MCF-7-LCC2 cells, TAM or MSA alone induced significant G1 accumulation (62.3% and 59.3%) compared with vehicle (39.3%; *, \( P < 0.05 \)) and combination of both agents induced even greater G1 accumulation (78.5%) compared with either agent alone (**, \( P < 0.05 \)). Apoptosis of MCF-7 (C) and MCF-7-LCC2 (D) cells after 36 h incubation with MSA (10 μmol/L), APC (1 and 2 μg), or MSA + APC. Representative of three or more identical experiments.
After incubation with a general caspase inhibitor, the apoptosis induced by both MSA and MSA + TAM was completely abrogated indicating that caspase-dependent pathways were required. Previous studies by Jiang et al. (21) have shown that caspases are essential executioners of MSA-induced apoptosis in DU-145 human prostate cancer cells. These authors examined the effects of MSA on the activation of multiple caspases (caspase-3, -7, -8, and -9), the release of cytochrome c from mitochondria, PARP cleavage, and DNA fragmentation. Their results indicated that MSA-induced apoptosis involved cell detachment as a prerequisite and was principally initiated by caspase-8 activation and subsequently amplified by its crosstalk with other caspases (caspase-9 and -3). In our experience with two ER-positive breast cancer cell lines incubated with MSA, we also observed cell detachment (data not shown) and caspase-8 activation in the apoptotic cells. However, caspase-9 was also activated by MSA and we found that selenium-induced caspase-8 activation required prior activation of caspase-9. These data are most consistent with a linear activation pathway from caspase-9 to caspase-8 (Fig. 6).

Caspase-8 is a key initiator caspase in the mitochondrial pathway of apoptosis. Mitochondrial damage results in the release of cytochrome c into the cytosol. Procaspase-9 is recruited and activated by cofactor Apaf-1 through a caspase recruitment domain, after cytochrome c binds to Apaf-1 together with dATP. Activated caspase-9 cleaves downstream executioner caspases to initiate the caspase cascade (32–35). Our finding of MSA-induced activation of caspase-9 suggests that MSA may act through the mitochondrial pathway. Similar to our study, Jiang et al. (21) have observed that MSA-induced apoptosis involved caspase-3- and -7-mediated PARP cleavage that was initiated by caspase-8 and amplified through caspase-9 activation and a feedback activation loop from caspase-3 in DU-145 prostate cancer cells. However, MCF-7 cells are caspase-3 deficient (36), indicating that the specific sequence of caspase activation in these cells is likely different than for DU-145 cells. Although several reports have shown caspase-9 activation following caspase-8 activation (37–39), it is also possible that caspase-8 may lie downstream of caspase-9. Because the half-lives of the cleaved (activated) caspases are very short, a positive feedback loop involving caspase-8 is required to amplify the apoptosis signal and maintain the signal for a duration long enough to execute the terminal apoptotic events (40). Li et al. (20) suggest that the crosstalk between intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways may be due to selenium-induced endoplasmic reticulum stress that causes a direct release of caspases from the endoplasmic reticulum followed by an indirect activation of both caspase-8 and -9. Our data showed that the caspase-8 inhibitor had no effect on cleavage of caspase-8 or -9. Conversely, caspase-9 inhibitor completely prevented cleavage of caspase-8. It should be noted that these inhibitors prevent caspase function but not the expression and cleavage of the caspase. Also, truncated BID, which is induced by the extrinsic pathway through caspase-8 to mediate crosstalk with the intrinsic pathway through activation of caspase-9 (41, 42), was not detected following treatment of MCF-7 and MCF-7-LCC2 cells with MSA (data not shown). Taken together, the findings from this report...
strongly suggest that apoptosis induced by MSA in breast cancer cells occurred via a linear pathway from caspase-9 to caspase-8 rather than the previously reported pathway from caspase-8, truncated BID, and then activation of caspase-9 (41, 42). Subsequent to caspase-8 activation, caspase-7 and then PARP cleavage led to selenium-induced DNA fragmentation and apoptosis in breast cancer cells (Fig. 6). Although our results do not exclude the ability of MSA to induce Fas ligand and "initiate" the extrinsic pathway, the data indicate that the activation of caspase-9 was essential for caspase-8 activation. It is possible that selenium initiation of extrinsic pathway cannot occur without prior selenium activation of caspase-9.

Although MSA induced similar levels of apoptosis in MCF-7 and MCF-7-LCC2 cells, activation of caspase-7 and -8 was much more robust in MCF-7-LCC2 compared with MCF-7 cells. It is possible that although both extrinsic and intrinsic apoptosis pathways occur in both cell lines in response to MSA, the extrinsic pathway is more robust in MCF-7-LCC2 than in MCF-7 cells. The modest MSA activation of the executioner caspase-7 in MCF-7 cells compared with MCF-7-LCC2 cells may indicate that low levels of the other executioner caspases-3 and -6 (undetectable by Western blot) may combine with low activation by caspase-7 in MCF-7.

Cell cycle analysis revealed interesting results in comparing tamoxifen-sensitive MCF-7 and tamoxifen-resistant MCF-7-LCC2 cells. As expected, TAM induced significant $G_1$ arrest in both MCF-7 and MCF-7-LCC2 cells. In MCF-7 cells, MSA alone exhibited very modest effects on $G_1$ arrest and the combination of MSA + TAM did not exhibit significantly greater $G_1$ arrest than TAM alone. In contrast, in MCF-7-LCC2 cells, both TAM and MSA alone induced significant $G_1$ arrest with the greatest $G_1$ arrest apparent when the two agents were combined.

This indicates that not only was a synergistic apoptosis occurring in tamoxifen-resistant MCF-7-LCC2 cells when the agents were combined but also a potentially synergistic $G_1$ arrest was operative.

Our previous study showed synergy of selenium and tamoxifen for growth inhibition in tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. The present study showed that this synergy occurred at least in part at the level of apoptosis induction. It is well known that the major growth-inhibitory mechanism for therapeutic levels of tamoxifen is cell cycle arrest (22, 23). At higher, supraphysiologic concentrations (>10^{-6} mol/L), tamoxifen may induce apoptosis (43). However, these concentrations are well above the concentration of tamoxifen required to fully saturate the ER in breast cancer cells. TAM at 10^{-7} mol/L did not result in apoptosis. Cell cycle analysis showed that TAM resulted in enhanced accumulation of cells in $G_1$. When combined with MSA, further $G_1$ arrest was found. In agreement with our study, Danova et al. (22) showed that tamoxifen induced significant $G_0$/$G_1$ arrest in breast cancer cells that was paralleled by a decrease in the frequency of cells expressing Ki-67 and proliferating cell nuclear antigen and by an increase in statin-positive ($G_0$) cells. Their results confirmed that tamoxifen-induced inhibition of cell growth is associated with major changes in the cell cycle variables of MCF-7 cells and also showed that two major mechanisms were operative: (a) the accumulation of cells in $G_1$ before the onset of S phase and (b) the exit of some cells from the cycling compartment. The likely mechanism for synergy between MSA and tamoxifen in apoptosis induction is the increased $G_1$ arrest induced by tamoxifen that allows more cells to enter the apoptosis pathway induced by selenium.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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