

# Selenium disrupts estrogen receptor $\alpha$ signaling and potentiates tamoxifen antagonism in endometrial cancer cells and tamoxifen-resistant breast cancer cells

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## Abstract

Tamoxifen, a selective estrogen receptor (ER) modulator, is the most widely prescribed hormonal therapy treatment for breast cancer. Despite the benefits of tamoxifen therapy, almost all tamoxifen-responsive breast cancer patients develop resistance to therapy. In addition, tamoxifen displays estrogen-like effects in the endometrium increasing the incidence of endometrial cancer. New therapeutic strategies are needed to circumvent tamoxifen resistance in breast cancer as well as tamoxifen toxicity in endometrium. Organic selenium compounds are highly effective chemopreventive agents with well-documented benefits in reducing total cancer incidence and mortality rates for a number of cancers. The present study shows that the organic selenium compound methylseleninic acid (MSA, 2.5  $\mu\text{mol/L}$ ) can potentiate growth inhibition of 4-hydroxytamoxifen ( $10^{-7}$  mol/L) in tamoxifen-sensitive MCF-7 and T47D breast cancer cell lines. Remarkably, in tamoxifen-resistant MCF-7-LCC2 and MCF7-H2 $\Delta$ 16 breast cancer cell lines and endometrial-derived HEC1A and Ishikawa cells, coinubation of 4-hydroxytamoxifen with MSA resulted in a marked growth inhibition that was substantially greater than MSA alone. Growth inhibition by MSA and MSA + 4-hydroxytamoxifen in all cell lines was preceded by a specific decrease in ER $\alpha$  mRNA and protein without an effect on ER $\beta$  levels. Estradiol and 4-hydrox-

ytamoxifen induction of endogenous ER-dependent gene expression (*pS2* and *c-myc*) as well as ER-dependent reporter gene expression (*ERE<sub>2</sub>e1b-luciferase*) was also attenuated by MSA in all cell lines before effect on growth inhibition. Taken together, these data strongly suggest that specific decrease in ER $\alpha$  levels by MSA is required for both MSA potentiation of the growth inhibitory effects of 4-hydroxytamoxifen and resensitization of tamoxifen-resistant cell lines. [Mol Cancer Ther 2005;4(8):1239–49]

## Introduction

Estrogens are key hormones for the growth and maintenance of female mammary gland and reproductive tract and critical for reproduction and fertility. Estrogen binds to its cognate receptor, the estrogen receptor (ER), belonging to the nuclear receptor superfamily of ligand-dependent transcription factors (1, 2). Two different forms of ER have been characterized, ER $\alpha$  and ER $\beta$ , which share high sequence homology (3–5). Upon ligand binding, the receptor undergoes conformational changes that release ER from chaperone proteins. Following dimerization, ER binds to estrogen-responsive elements (ERE) in the promoters of ER-dependent genes, and subsequent recruitment of coactivators initiates ER-dependent gene transcription (6, 7).

In addition to maintaining normal reproductive physiology, estrogens are important mitogenic signals in the breast and endometrium thus implicating the hormone in breast and endometrial tumorigenesis. As a treatment for breast cancer, tamoxifen a selective ER modulator, binds to ER and blocks estrogen-mediated breast cancer cell growth (8, 9). Tamoxifen is the most widely prescribed endocrine therapy for breast cancer and the only agent approved for breast cancer chemoprevention (10). However, tamoxifen therapy has two major drawbacks. Most tamoxifen-responsive breast cancer patients succumb to tamoxifen resistance (11) in which tumors do not respond to the growth inhibitory properties of tamoxifen. In addition, tamoxifen displays estrogen-like effects in the endometrium increasing the incidence of endometrial cancer (12). Alternative therapeutic strategies that can be used alone or in combination with tamoxifen in ER $\alpha$ -positive breast cancers may prove useful in combating tamoxifen resistance in breast and estrogenic activities in other tissues.

Selenium is an essential micronutrient shown to inhibit cancer growth. Organic selenium compounds are the agents of choice for chemopreventive studies. These compounds have fewer side effects and lack the genotoxic

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action of inorganic selenium compounds such as selenite (13). Organic selenium agents used in chemoprevention trials such as methylselenocysteine and seleno-L-methionine are water-soluble compounds that are metabolized in tissues to the active selenium metabolite, methylselenol (14–16). The clinical usefulness of methylselenocysteine and seleno-L-methionine are in the ability of these compounds to inhibit DNA synthesis and cell doubling and induce apoptotic cell death. One of the greatest benefits of organic selenium compounds for chemoprevention is the very low or absent toxicity (16, 17).

In a double-blind placebo-controlled clinical trial, Clark et al. showed the protective effects of selenium-enriched yeast against prostate, lung, and colon cancer (16, 18). Although, the study failed to show statistical significance in breast cancer risk due to insufficient cases, there is extensive data showing the growth inhibitory properties of selenium in breast cancer cell lines and mammary tumor models. Our previous study using methylseleninic acid (MSA), a rapidly metabolized selenium compound useful in cell cultures studies (19), showed that MSA inhibits estradiol induced cell growth and ER $\alpha$ -mediated gene transcription in the ER $\alpha$ -positive MCF-7 breast cancer cell line with no significant toxicity.<sup>4</sup> The major mechanism by which MSA attenuates ER signaling was through decrease in ER $\alpha$  mRNA levels and subsequent protein levels with no effect on ER $\beta$  levels. These data suggested a novel mechanism of growth inhibition by MSA through disruption of estrogen signaling.

Because breast cancers vary widely with regard to ER $\alpha$  expression and *de novo* tamoxifen resistance, the present study examined the growth inhibitory mechanisms of MSA in cell lines that represent different paradigms of ER $\alpha$  expression and tamoxifen sensitivity/resistance. We show that MSA can inhibit ER signaling and potentiate the antiestrogen activity of tamoxifen via down-regulation of ER $\alpha$  mRNA and protein levels. MSA in combination with tamoxifen potentiated growth inhibitory properties when compared with either agent alone in tamoxifen-sensitive breast cancer cell lines and tamoxifen-resistant breast cell lines and endometrial cell lines where tamoxifen displays agonist activity.

## Materials and Methods

### Cell Lines

MCF-7 (tamoxifen-sensitive, ER-positive breast cancer cell line), MCF7-H2 $\Delta$ 16 (tamoxifen-resistant, ER-positive MCF-7 variant overexpressing mutant ErbB2<sup>5</sup>), MDA-MB-231 (tamoxifen-resistant, ER-negative breast cancer cell line), and MDA-MB-468 (tamoxifen-resistant, ER-negative

breast cancer cell line) were maintained in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY), 4 mmol/L of glutamine (Life Technologies), and 1% penicillin-streptomycin (Life Technologies) at 37°C with 5% CO<sub>2</sub>. T47D (tamoxifen-sensitive, ER-positive breast cancer cell line) was maintained RPMI 1640 (Life Technologies) supplemented 10% FBS, 1% penicillin-streptomycin, and 5  $\mu$ g/mL of insulin (Sigma, St. Louis, MO). MCF-7-LCC2 (tamoxifen-resistant, ER-positive breast cancer cell line) was maintained in IMEM (Biosource, Rockville, MD) supplemented with 5% FCS (Life Technologies) that had been charcoal stripped to remove endogenous steroids and 1% penicillin-streptomycin at 37°C with 5% CO<sub>2</sub>. Ishikawa (tamoxifen-agonist, ER-positive endometrial cancer cell line) and HEC1A (tamoxifen-agonist, ER-positive endometrial cancer cell line) were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin at 37°C with 5% CO<sub>2</sub>.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used for the quantitative measurement of cellular growth. MTT salts are cleaved to formazan by the succinate-tetrazolium reductase enzymes, which are only active in viable cells. MCF-7, T47D, MCF-7-LCC2, MCF7-H2 $\Delta$ 16, MDA-MB-231, MDA-MB-468, Ishikawa, and HEC1A cells were plated in 24-well plates (20,000 cells per well) and cultured in medium described above. MCF-7, T47D, MCF-7-LCC2, Ishikawa, and HEC1A cells were incubated with MSA (2.5  $\mu$ mol/L), 4-hydroxytamoxifen (10<sup>-7</sup> mol/L, Sigma), or combination of 4-hydroxytamoxifen and MSA at 24 hours after plating. MCF7-H2 $\Delta$ 16, MDA-MB-231, and MDA-MB-468 were incubated with MSA (1  $\mu$ mol/L), 4-hydroxytamoxifen (10<sup>-7</sup> mol/L, Sigma), or combination of 4-hydroxytamoxifen and MSA at 24 hours after plating. An aliquot of 125  $\mu$ L of MTT reagent (ICN, Aurora, OH; 5 mg/mL of 2,5-diphenyl tetrazolium bromide in PBS) was pipetted into each well after 24, 48, 72, or 96 hours posttreatment. The medium with the MTT reagent was removed after 30 minutes to 1 hour and 300  $\mu$ L of DMSO (Fisher Biotech, Fairlawn, NJ) were added to each well. The plates were read at a wavelength of 570 nm.

### Luciferase Assay

MCF-7, Ishikawa, and HEC1A were plated in 6-well plates (2  $\times$  10<sup>5</sup> cells per well) and cultured in phenol red-free DMEM containing 2% charcoal-stripped FBS. MCF-7-LCC2 were plated in 6-well plates (2  $\times$  10<sup>5</sup> cells per well) cultured in phenol red-free IMEM containing 2% charcoal-stripped FCS. 24 hours after plating; MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A cells were transfected with 500 ng of *EREe1b-luciferase* reporter using Fugene transfection reagent (Roche, Madison, WI). Twenty-four hours after transfection, MCF-7, Ishikawa, and HEC1A cells were incubated with vehicle, estradiol (10<sup>-8</sup> mol/L, Sigma), 4-hydroxytamoxifen (10<sup>-7</sup> mol/L), MSA (10  $\mu$ mol/L), or various combinations of estradiol, tamoxifen, and MSA for

<sup>4</sup> Y.M. Shah, et al. Attenuation of Estrogen Receptor  $\alpha$  (ER $\alpha$ ) Signaling by Selenium in Breast Cancer Cells via Downregulation of ER $\alpha$  Gene Expression. *Breast Cancer Research and Treatment*, in press.

<sup>5</sup> F. Jones, et al. An oncogenic isoform of ERBB2/HER2 associated with metastatic breast cancer, submitted for publication.

24 hours. MCF-7-LCC2 were incubated with vehicle, estradiol ( $10^{-9}$  mol/L), 4-hydroxytamoxifen ( $10^{-8}$  mol/L), MSA (10  $\mu$ mol/L), or various combinations of estradiol, tamoxifen and MSA for 24 hours. Luciferase expression was measured and normalized as previously described (20).

#### Western Blot Analysis

MCF-7, MCF-7-LCC2, and Ishikawa were plated in 100-mm dishes ( $3 \times 10^6$  cells per plate) and cultured in 2% charcoal-stripped FBS in DMEM. MCF-7-LCC2 were plated in 100-mm dishes ( $3 \times 10^6$  cells per plate) and cultured in 2% charcoal-stripped FCS in IMEM. The cells were maintained in the stripped media for 3 days until 90% confluency. MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A were incubated with vehicle, estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), MSA (10  $\mu$ mol/L), or a combination of estradiol or 4-hydroxytamoxifen and MSA for 6 hours. The cells were lysed and prepared for Western blotting as previously described (20). The membranes were incubated with an antibody against ER $\alpha$  (Novacastra, Newcastle upon Tyne, United Kingdom) and normalized to  $\beta$ -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

#### Real-time Reverse Transcription-PCR

The culture conditions for MCF-7, MCF-7-LCC2, Ishikawa, and HEC1A were identical to that described above for Western blot analysis. The cells were incubated with vehicle, estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), MSA (10  $\mu$ mol/L), or a combination of estradiol or tamoxifen and MSA for 2 hours when measuring ER $\alpha$  and ER $\beta$  mRNA expression and 6 hours when measuring *c-myc* and *p52* mRNA expression. Total mRNA was extracted from the cell pellet, reverse transcribed, and gene expression was measured by real-time reverse transcription-PCR as described previously (20). *c-myc*: forward 5'-CGTCTCCACACATCAGCACAA-3', reverse 5'-TGTTGGCAGCAGGATAGTCCTT-3', probe 5'-56FAM/ACGCAGCGCCTCCCTCCACTC/3BHQ-1/-3'; *p52*: forward 5'-CGTCAAAGACAGAATTGTGGTTTT-3', reverse 5'-CGTCGAAACAGCAGCCCTTA-3', probe 5'-56FAM/TGTCACGCCCTCCAGTGTGCA/3BHQ-1/-3'; ER $\alpha$ : forward 5'-AGACGGACCAAAGCCACTTG-3', reverse 5'-CCCCGTGATGTAATACTTTTGA-3', probe 5'-56FAM/TGCGGGCTCTACTTCATCGCATTCC/3BHQ-1/-3'; ER $\beta$ : forward 5'-CCCAGTCGCGCCCTTAC-3', reverse 5'-CAACTCCTTGTCGGCCAACT-3', probe 5'-56FAM/AGGCCTCCATGATGTCCCTGA/3BHQ-1/-3'.

#### Ligand Binding Assay

MCF-7 cells were plated in 6-well plates ( $2 \times 10^5$  cells per plate) and cultured in 2% charcoal-stripped FBS in DMEM. The cells were maintained in the stripped media for 3 days until 90% confluent. MCF-7 cells were incubated with MSA (10  $\mu$ mol/L) for 1 hour. Following 1-hour incubation, 10 nmol/L [ $^3$ H] E $_2$  in the presence or absence of 500-fold excess cold estradiol were added to each well and incubated for 1 hour. Following 1-hour incubation, the cells were washed thrice with cold  $1 \times$  PBS. Ethanol (700  $\mu$ L) was added to each well and incubated at room temperature for 30 minutes. Ethanolic extract (500  $\mu$ L) was

counted using liquid scintillation. The relative binding affinity was calculated by (specific binding – nonspecific binding / specific binding)  $\times$  100. Vehicle-treated samples were set as 1.

#### Data Analysis

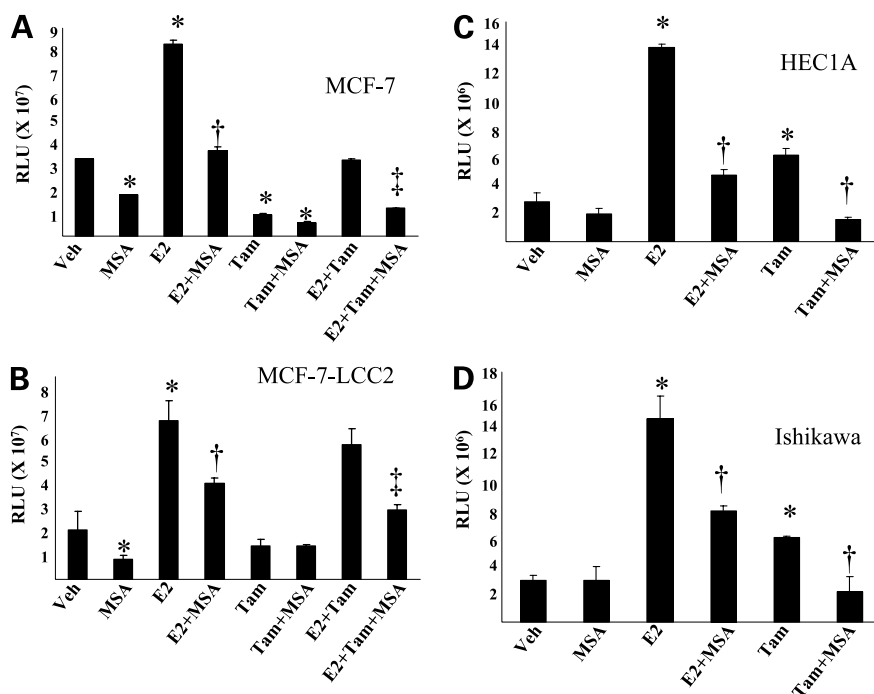
Results are expressed as mean  $\pm$  SD. *P* values were calculated using ANOVA, Dunnett's *t* test, and independent *t* test. *P* < 0.05 was considered significant.

## Results

### MSA Inhibits Estradiol- and Tamoxifen-Dependent Activation of an ERE-Luciferase Reporter Gene (*ERE<sub>2</sub>e1b-luciferase*)

The effects of MSA on ER signaling were assessed in MCF-7 cells, a well-characterized ER-positive, tamoxifen-sensitive human breast cancer cell and in MCF-7-LCC2 cells, an ER-positive, tamoxifen-resistant variant of the MCF-7 parental line. Two human endometrial cancer cell lines, Ishikawa and HEC1A, were also assessed for MSA effects on the *ERE<sub>2</sub>e1b-luciferase*. MCF-7, HEC1A, and Ishikawa cells were transfected with the *ERE<sub>2</sub>e1b-luciferase* reporter gene and incubated with estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), or MSA (10  $\mu$ mol/L) alone, or coincubated with combinations of estradiol, 4-hydroxytamoxifen, and MSA as indicated (Fig. 1A, C, and D). MCF-7-LCC2 cells were transfected with the *ERE<sub>2</sub>e1b-luciferase* reporter gene and incubated with estradiol ( $10^{-9}$  mol/L), 4-hydroxytamoxifen ( $10^{-8}$  mol/L), or MSA (10  $\mu$ mol/L) alone, or coincubated with combinations of estradiol, 4-hydroxytamoxifen, and MSA as indicated (Fig. 1B). Although MCF-7-LCC2 cells are resistant to tamoxifen growth inhibition, the cells do not display a true tamoxifen agonist activation of the *ERE<sub>2</sub>e1b-luciferase* reporter (Fig. 1B, column 5), an activation that is observed in the endometrial cancer cell lines HEC1A and Ishikawa (Fig. 1C-D, column 5) as we and others have previously reported (20–22). Tamoxifen resistance in MCF-7-LCC2 cells is manifested by the inability of 4-hydroxytamoxifen to reverse estradiol activation of the reporter (Fig. 1B, column 7), which is observed at  $10^{-9}$  mol/L estradiol +  $10^{-8}$  mol/L 4-hydroxytamoxifen. In the tamoxifen-sensitive MCF-7 parental cells, a higher concentration of estradiol ( $10^{-8}$  mol/L) was required to observe estradiol activation of the reporter. Consequently, a higher concentration of 4-hydroxytamoxifen ( $10^{-7}$  mol/L) was used for reversing estradiol activation in MCF-7 cells (Fig. 1A, column 7).

To determine whether MSA might affect reporter activation by estradiol or tamoxifen, cells were coincubated with estradiol, 4-hydroxytamoxifen, and MSA. MSA alone decreased basal *ERE<sub>2</sub>e1b-luciferase* expression in MCF-7 and MCF-7-LCC2 cells (Fig. 1A-B, column 2) and inhibited estradiol-dependent stimulation of the reporter in all cell lines (Fig. 1A-D, columns 3 and 4). Although MSA did not significantly affect 4-hydroxytamoxifen action in either breast cancer cell line (Fig. 1A and B, column 6), MSA completely blocked 4-hydroxytamoxifen activation of the reporter in both endometrial cell lines (Fig. 1C and D,



**Figure 1.** MSA inhibits estrogen and tamoxifen-activated *ERE<sub>2</sub>e1b-luciferase* reporter. MCF-7 (A), MCF-7-LCC2 (B), HEC1A (C), and Ishikawa (D) cells ( $2 \times 10^5$  cells per well) were transfected with *ERE<sub>2</sub>e1b-luciferase* reporter (0.5  $\mu$ g per well). Twenty-four h after transfection, cells were incubated with vehicle (Veh);  $10^{-8}$  mol/L estradiol (E2) for MCF-7, HEC1A, and Ishikawa or  $10^{-9}$  mol/L estradiol for MCF-7-LCC2 cells;  $10^{-7}$  mol/L 4-hydroxytamoxifen (Tam) for MCF-7, HEC1A, and Ishikawa or  $10^{-8}$  mol/L 4-hydroxytamoxifen for MCF-7-LCC2 cells; or 10  $\mu$ mol/L MSA alone; or coincubated with estradiol and MSA, 4-hydroxytamoxifen, and MSA, or estradiol, 4-hydroxytamoxifen, and MSA for 24 h. Standard luciferase assays were done on cell extracts in as described in Materials and Methods. The data is presented as relative light units (RLU) defined as the luciferase output normalized to protein content. Columns, means; bars,  $\pm$ SD. \*,  $P < 0.05$ , compared with vehicle-incubated samples. †,  $P < 0.05$ , compared with estradiol- or 4-hydroxytamoxifen-treated samples. ‡,  $P < 0.05$ , compared with estradiol- and 4-hydroxytamoxifen-coincubated samples.

column 6). Cotreatment with MSA potentiated the inhibitory effect of 4-hydroxytamoxifen on estradiol activation of the reporter in MCF-7 cells (Fig. 1A, compare columns 7 and 8). In MCF-7-LCC2 cells, MSA combined with tamoxifen reversed tamoxifen resistance by blocking estradiol activation of the reporter greater than the effect of MSA alone (Fig. 1B, compare columns 4, 7, and 8). Note that coincubation with 4-hydroxytamoxifen + estradiol + MSA resulted in greater inhibition of reporter activation than estradiol + MSA (Fig. 1A-B, compare columns 4 and 8) or estradiol + 4-hydroxytamoxifen (Fig. 1A-B, compare columns 6 and 8). These data show that MSA inhibits estradiol activation of the *ERE<sub>2</sub>e1b-luciferase* reporter in breast and endometrial cancer cells, potentiates the anti-estrogen effects of 4-hydroxytamoxifen in MCF-7-LCC2 cells, and blocks 4-hydroxytamoxifen agonist action in endometrial cancer cell lines.

#### MSA Inhibits the Endogenous ER $\alpha$ -Regulated Gene Expression

To determine whether MSA affected endogenous ER-regulated genes, the well-characterized estrogen-regulated gene *c-myc* was assessed. MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa were incubated with either vehicle, estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), or MSA (10  $\mu$ mol/L) alone or a combination of estradiol or 4-hydroxytamoxifen with MSA for 6 hours (Fig. 2A-D). MSA had no effect on basal *c-myc* gene expression with the exception of HEC1A cells (Fig. 2A-D, column 2). However, MSA inhibited estradiol-induced gene expression of *c-myc* in all cell lines (Fig. 2A-D, columns 3 and 4). 4-Hydroxytamoxifen had no effect on basal *c-myc* gene expression in MCF-7 cells, and MSA in combination with 4-hydroxytamoxifen also had no effect when compared

with vehicle or 4-hydroxytamoxifen-incubated samples alone (Fig. 2A, columns 5 and 6). In contrast to the inability of 4-hydroxytamoxifen to activate the *ERE<sub>2</sub>e1b-luciferase* reporter in MCF-7-LCC2 cells, 4-hydroxytamoxifen displayed true agonist activation of endogenous *c-myc* in these cells (Fig. 2B, column 5) that was also evident in both endometrial cell lines HEC1A and Ishikawa (Fig. 2C-D, column 5). MSA blocked 4-hydroxytamoxifen activation of *c-myc* in MCF-7-LCC2, Ishikawa, and HEC1A cells (Fig. 2B-D, column 6). Similar results to those described in Fig. 2 were found for the ER-dependent *p52* gene (data not shown).

#### MSA Reduces ER $\alpha$ Protein Levels in Tamoxifen-Sensitive and -Resistant Cell Lines

We previously showed that ER $\alpha$  protein and mRNA down-regulation was likely a major mechanism by which MSA inhibited ER signaling in MCF-7 cells; MSA had no effect on ER $\beta$  mRNA.<sup>4</sup> These experiments were extended to determine whether MSA altered ER $\alpha$  protein in both tamoxifen-sensitive and -resistant cells. In addition, the effect of estradiol or 4-hydroxytamoxifen alone or in combination with MSA on ER $\alpha$  protein expression was also assessed. Due to very low expression of ER $\alpha$  in HEC1A cells, the Western blot analysis was inconclusive (data not shown). MCF-7, MCF-7-LCC2, and Ishikawa cells were incubated with estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), or MSA (10  $\mu$ mol/L) alone, or estradiol or 4-hydroxytamoxifen in combination with MSA for 6 hours and ER $\alpha$  levels were assessed by Western blot analysis. Estradiol treatment alone reduced ER $\alpha$  protein levels only in the MCF-7 cells (Fig. 3A, column 3) that is likely mediated through the ubiquitin proteasome pathway (23). In contrast to estradiol, MSA alone significantly reduced ER $\alpha$  protein

in all cell lines (Fig. 3A-C, column 2). MSA further reduced ER $\alpha$  levels when coincubated with estradiol in MCF-7 cells (Fig. 3A, columns 3 and 4) as we have previously shown.<sup>4</sup> Remarkably, in MCF-7-LCC2 and Ishikawa cells, estradiol had no effect on ER $\alpha$  protein expression, a previously unreported observation for estradiol regulation of ER $\alpha$  (Fig. 3B and C, column 3). Estradiol + MSA reduced ER $\alpha$  protein expression to the level detected with MSA treatment alone (Fig. 3B and C, columns 3 and 4).

4-Hydroxytamoxifen stabilized ER $\alpha$  protein expression in MCF-7 cells (Fig. 3A, column 5) as previously reported (24). 4-Hydroxytamoxifen also stabilized ER $\alpha$  expression in Ishikawa cells but had no effect on ER $\alpha$  levels in MCF-7-LCC2 cells (Fig. 3B and C, column 5). Coincubation of 4-hydroxytamoxifen with MSA decreased ER $\alpha$  expression when compared with either vehicle or 4-hydroxytamoxifen alone in all cell lines (Fig. 3A-C, columns 5 and 6).

Taken together, these data show that MSA down-regulation of ER $\alpha$  is not restricted to tamoxifen-sensitive MCF-7 cells and occurs in the presence or absence of ligand. Two unrelated findings from these experiments were that estradiol had no effect on ER $\alpha$  protein expression in MCF-7-LCC2 and Ishikawa cells and that 4-hydroxytamoxifen stabilized ER $\alpha$  expression in Ishikawa cells (Fig. 3C, column 5) but had no effect on ER $\alpha$  levels in MCF-7-LCC2 cells (Fig. 3B, column 5). The molecular mechanisms of cell-specific and ligand-dependent receptor turnover are currently being investigated.

#### MSA Reduces ER $\alpha$ mRNA but Has No Effect on ER $\beta$

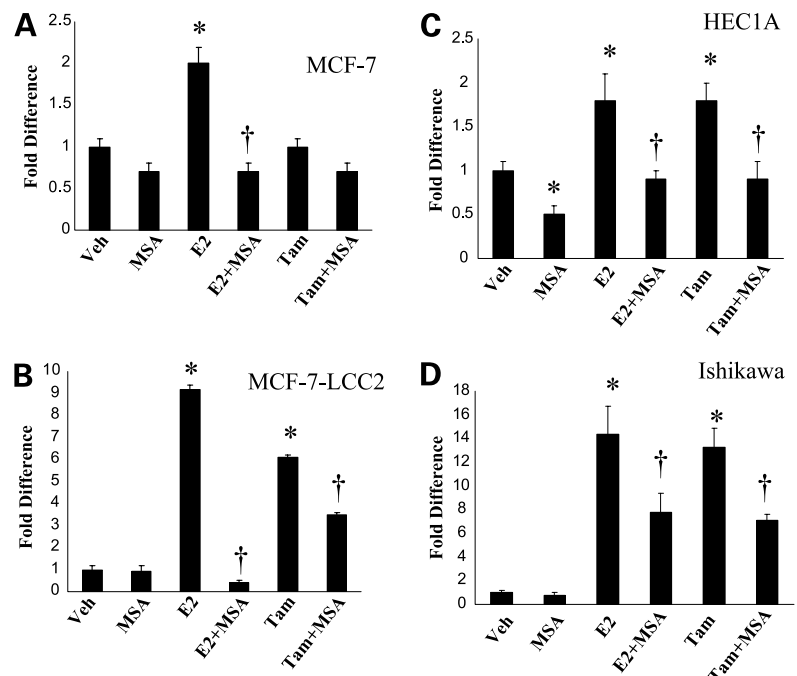
Our previous study found that MSA decreased ER $\alpha$  mRNA in MCF-7 cells and the decrease in mRNA preceded a decrease in ER $\alpha$  protein.<sup>4</sup> We extended these studies to tamoxifen-resistant and endometrial cell lines and also

included assessment of 4-hydroxytamoxifen + MSA. ER $\alpha$  mRNA in MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa cells was measured by real-time reverse transcription-PCR following incubation of cells with estradiol ( $10^{-8}$  mol/L), 4-hydroxytamoxifen ( $10^{-7}$  mol/L), or MSA ( $10 \mu\text{mol/L}$ ) alone or estradiol or 4-hydroxytamoxifen + MSA for 2 hours. As previously shown, MSA reduced ER $\alpha$  expression in MCF-7 cells (Fig. 4A, column 2). MSA also decreased ER $\alpha$  mRNA in MCF-7-LCC2, HEC1A, and Ishikawa cells (Fig. 4B-C, column 2). Estradiol or 4-hydroxytamoxifen had no effect on ER $\alpha$  gene expression in MCF-7, MCF-7-LCC2, and HEC1A (Fig. 4A-C, columns 3 and 5), although 4-hydroxytamoxifen significantly decreased ER $\alpha$  mRNA in Ishikawa cells (Fig. 4D, column 5). Coincubation of estradiol or 4-hydroxytamoxifen with MSA decreased ER $\alpha$  mRNA to levels observed with MSA alone (Fig. 4A-D, columns 4 and 6). MSA, estradiol, or 4-hydroxytamoxifen alone or in combination had no effect on ER $\beta$  mRNA expression in all cell lines (Fig. 5A-D) suggesting selective effects of MSA on ER $\alpha$ .

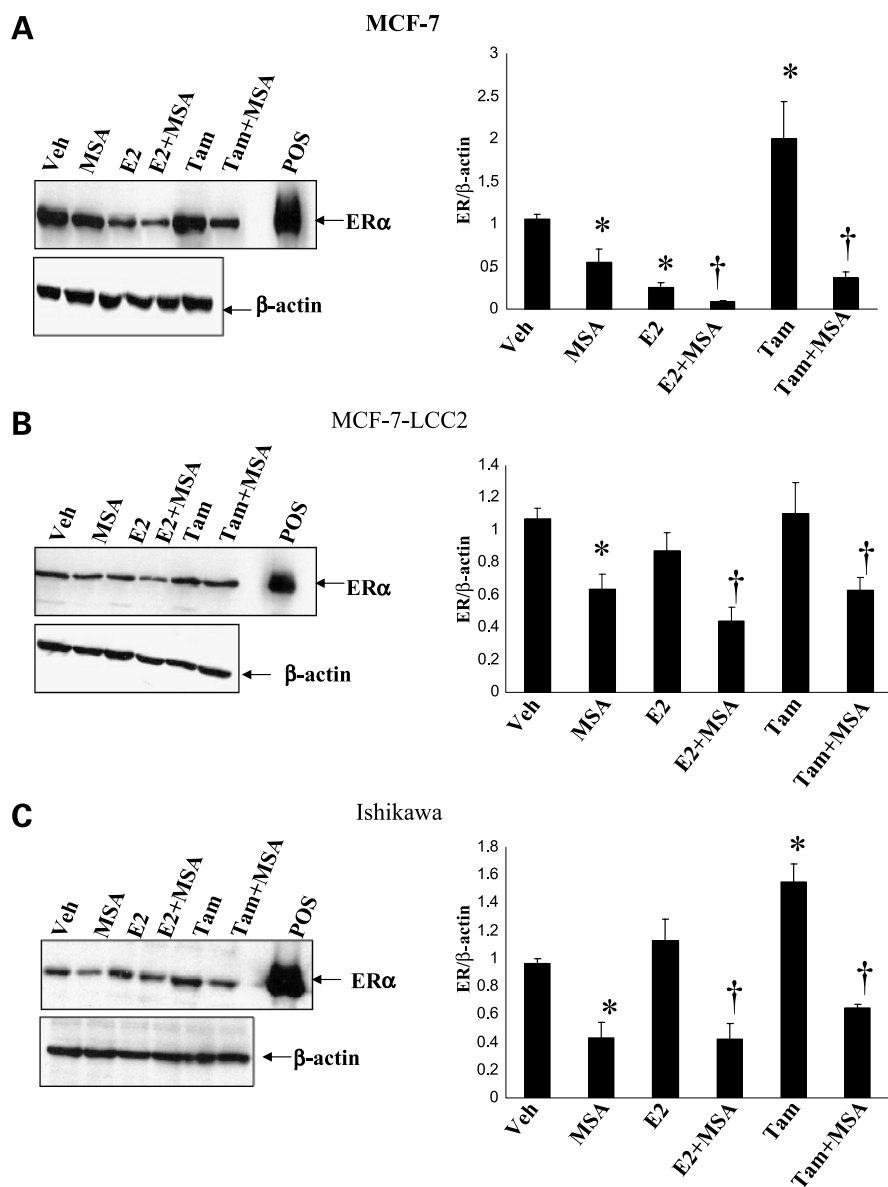
At a 2-hour incubation with MSA, ER $\alpha$  mRNA was reduced with no observed effects on ER $\alpha$  protein levels (data not shown). This suggests that the MSA-dependent decrease in ER $\alpha$  mRNA may account for subsequent decrease in ER $\alpha$  protein. Remarkably, although 4-hydroxytamoxifen decreased ER $\alpha$  mRNA in Ishikawa cells (Fig. 4D, column 5), ER $\alpha$  protein actually increased with treatment (Fig. 3D, column 5). The mechanisms underlying this novel observation are under investigation.

#### MSA Potentiates the Growth Inhibitory Properties of 4-Hydroxytamoxifen

Our previous study found that MSA decreased the growth of MCF-7 cells through a combination of decreased



**Figure 2.** MSA inhibits estradiol- and tamoxifen-dependent activation of *c-myc* gene expression. MCF-7 (A), MCF-7-LCC2 (B), HEC1A (C), and Ishikawa (D) cells ( $2 \times 10^6$  cells per plate) were incubated with vehicle (Veh),  $10^{-8}$  mol/L estradiol (E2),  $10^{-7}$  mol/L 4-hydroxytamoxifen (Tam), or  $10 \mu\text{mol/L}$  MSA alone, or coincubated with estradiol and MSA or 4-hydroxytamoxifen and MSA for 6 h. *c-myc* mRNA was measured by real-time reverse transcription-PCR as described in Materials and Methods. Expression was normalized to GAPDH. Columns, means; bars,  $\pm$ SD. \*,  $P < 0.05$ , compared with vehicle-incubated samples. †,  $P < 0.05$ , compared with estradiol- or 4-hydroxytamoxifen-incubated samples.



**Figure 3.** MSA reduces ER $\alpha$  protein. MCF-7 (A), MCF-7-LCC2 (B), and Ishikawa (C) cells ( $2 \times 10^6$  cells per plate) were incubated with vehicle (Veh),  $10^{-8}$  mol/L estradiol (E2),  $10^{-7}$  mol/L 4-hydroxytamoxifen (Tam), or  $10 \mu\text{mol/L}$  MSA alone, or coincubated with estradiol and MSA or 4-hydroxytamoxifen and MSA for 6 h. Western blot analysis was done. Quantitation of the Western blot signal for ER $\alpha$  was normalized to the Western blot signal for  $\beta$ -actin levels (A, B, and C, right). Columns, means; bars,  $\pm$ SD. \*,  $P < 0.05$ , compared with vehicle-incubated samples. †,  $P < 0.05$ , compared with estradiol- or 4-hydroxytamoxifen-incubated samples.

DNA synthesis and elevated apoptosis.<sup>4</sup> MCF-7 cells are also sensitive to growth inhibition by tamoxifen (25). It was of interest to determine whether coincubation of 4-hydroxytamoxifen with MSA could further potentiate growth inhibition compared with either agent alone. Furthermore, because long-term tamoxifen treatment is associated with tamoxifen resistance and endometrial proliferation, it was desirable to know whether coincubation of MSA + 4-hydroxytamoxifen could reverse tamoxifen resistance in breast cancer cells and inhibit tamoxifen-induced endometrial cell proliferation. MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa were incubated with 4-hydroxytamoxifen ( $10^{-7}$  mol/L), MSA ( $2.5 \mu\text{mol/L}$ ), or both agents for 24, 48, 72, and 96 hours and growth was assessed by the MTT assay. An additional tamoxifen-sensitive ER-positive breast cancer cell line, T47D and a tamoxifen-

resistant MCF-7 variant cell line overexpressing a mutant ErbB2, MCF7-H2 $\Delta$ 16, were also assessed. Only tamoxifen-sensitive MCF-7 and T47D cells were growth inhibited by 4-hydroxytamoxifen, whereas no effect was observed in the tamoxifen-resistant MCF-7-LCC2 and MCF7-H2 $\Delta$ 16 and endometrial-derived HEC1A and Ishikawa cell lines (Fig. 6A). Increasing incubation time with 4-hydroxytamoxifen to 9 days resulted in proliferation of HEC1A and Ishikawa cells (20). In all cell lines, MSA decreased cell growth by 96 hours of incubation (Fig. 6B-G). In tamoxifen-sensitive MCF-7 and T47D cells, MSA potentiated 4-hydroxytamoxifen effects on reducing cell growth with the combined treatment more effective than either agent alone (Fig. 6B and C). Remarkably, in tamoxifen-resistant MCF-7-LCC2 and MCF7-H2 $\Delta$ 16 and endometrial-derived HEC1A and Ishikawa cells in which 4-hydroxytamoxifen

alone has no effect on cell growth, cocubation of 4-hydroxytamoxifen with 1  $\mu\text{mol/L}$  MSA for MCF7-H2 $\Delta$ 16 cells and 2.5  $\mu\text{mol/L}$  MSA for HEC1A and Ishikawa cells resulted in a marked decrease in cell growth that was substantially greater than MSA treatment alone (Fig. 6D-G). These results show that MSA not only potentiates the antiestrogen effect of 4-hydroxytamoxifen in tamoxifen-sensitive cells, but MSA also resensitizes tamoxifen-resistant cells to the growth inhibitory properties of 4-hydroxytamoxifen.

#### Tamoxifen and MSA Do Not Synergize for Growth Inhibition in ER $\alpha$ -Negative Cell Lines

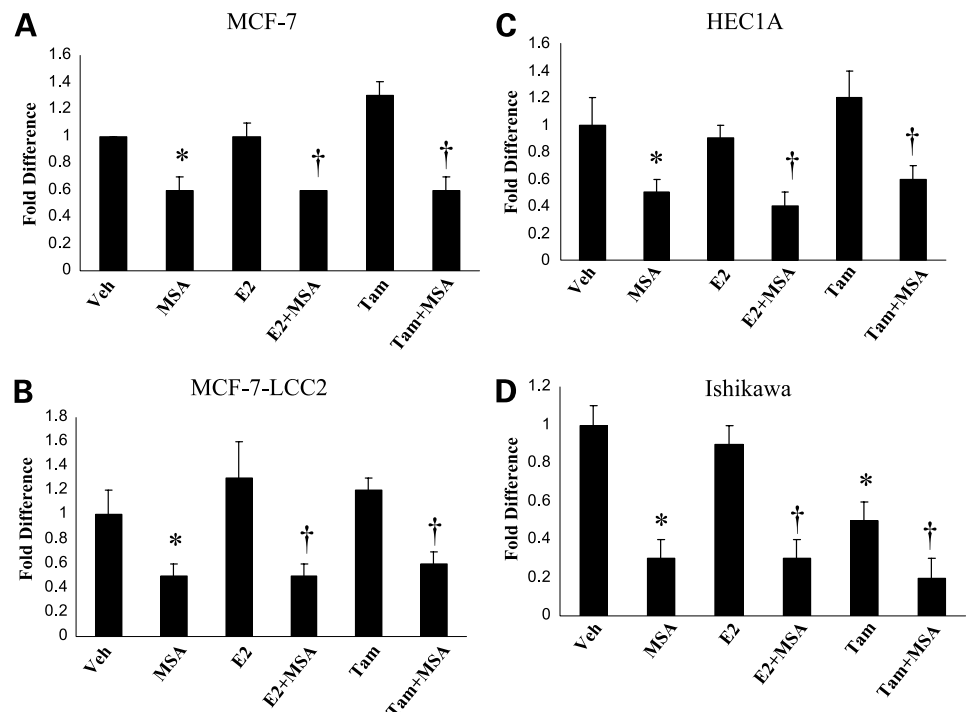
MDA-MB-231 and MDA-MB-468 cells, ER $\alpha$ -negative (26) and ER $\beta$ -positive (27) human breast cancer cell line, were incubated with 4-hydroxytamoxifen ( $10^{-8}$  mol/L) for 24, 48, 72, and 96 hours and growth was assessed by the MTT assay. No growth inhibitory effects were observed after incubation with 4-hydroxytamoxifen (Fig. 7A and B) as previously reported (28). Although MSA (1  $\mu\text{mol/L}$ ) alone decreased cell growth (Fig. 7A and B), cocubation of 4-hydroxytamoxifen with MSA did not further decrease cell growth (Fig. 7A and B) suggesting that ER $\alpha$  may be required for the additive and/or synergistic effect of tamoxifen + MSA on cell growth.

Inorganic selenite is reported to interact with the ligand binding domain of ER $\alpha$  and activate ER $\alpha$  transcriptional activity (29). To determine whether MSA altered estradiol binding to ER $\alpha$ , whole cell ligand binding assays were done in MCF-7 cells. One-hour incubation of MCF-7 cells with MSA (10  $\mu\text{mol/L}$ ) did not alter [ $^3\text{H}$ ] estradiol binding to ER $\alpha$  (Fig. 7C).

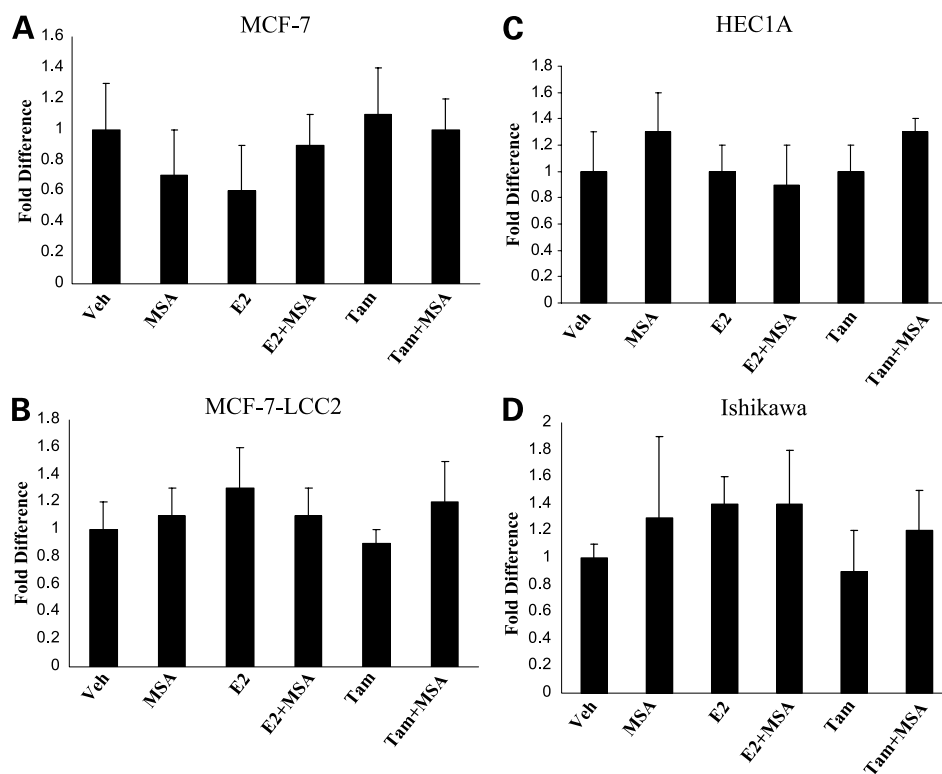
#### Discussion

Tamoxifen resistance and tamoxifen-induced endometrial proliferation are major limitations of tamoxifen therapy and chemoprevention. The present study shows that MSA inhibition of ER $\alpha$  signaling is not restricted to tamoxifen-sensitive MCF-7 cells. MSA antagonism of estradiol-dependent *ERE<sub>2</sub>e1b-luciferase* and endogenous *c-myc* and *p52* gene expression was shown in MCF-7-LCC2 cells, an ER $\alpha$ -positive, tamoxifen-resistant variant of the MCF-7 parental line and in two ER $\alpha$ -positive human endometrial cancer cell lines, Ishikawa and HEC1A (Figs. 1 and 2). In addition, MSA also blocked tamoxifen activation of these genes in endometrial Ishikawa and HEC1A cells (Fig. 1 and Fig. 2C-D, column 6). The major mechanism for MSA disruption of ER signaling in all ER $\alpha$ -positive cells lines was via rapid decrease of ER $\alpha$  mRNA and protein that preceded disruption of ER $\alpha$ -regulated gene expression (Figs. 3 and 4). MSA alone inhibited the growth of tamoxifen-sensitive, tamoxifen-resistant and endometrial-derived cells. MSA also potentiated tamoxifen growth inhibition of ER $\alpha$ -positive, tamoxifen-sensitive cells (MCF-7 and T47D) and tamoxifen-resistant cells (MCF-7-LCC2 and MCF7-H2 $\Delta$ 16) and endometrial-derived cells (Ishikawa and HEC1A; Fig. 6) but not ER $\alpha$ -negative, tamoxifen-resistant cells (MDA-MB-231 and MDA-MB-468; Fig. 7A) suggesting that ER $\alpha$  is required for the additive and/or synergistic effect on cell growth inhibition when MSA is combined with tamoxifen.

Interestingly, our studies showed that sensitivity to MSA growth inhibition is cell line specific. HEC1A and MCF7-H2 $\Delta$ 16 exhibited a marked decrease in cell growth after



**Figure 4.** MSA reduces ER $\alpha$  mRNA. MCF-7 (A), MCF-7-LCC2 (B), HEC1A (C), and Ishikawa (D) cells ( $2 \times 10^6$  cells per plate) were incubated with vehicle (Veh),  $10^{-8}$  mol/L estradiol (E2),  $10^{-7}$  mol/L 4-hydroxytamoxifen (Tam), or 10  $\mu\text{mol/L}$  MSA alone, or cocubated with estradiol and MSA or 4-hydroxytamoxifen and MSA for 2 h. ER $\alpha$  mRNA was measured by real-time RT-PCR as described in Materials and Methods. Expression was normalized to GAPDH. Columns, means; bars,  $\pm$ SD. \*,  $P < 0.05$ , compared with vehicle-incubated samples. †,  $P < 0.05$ , compared with estradiol- or 4-hydroxytamoxifen-incubated samples.



**Figure 5.** MSA does not alter ER $\beta$  mRNA. MCF-7 (A), MCF-7-LCC2 (B), HEC1A (C), and Ishikawa (D) cells ( $2 \times 10^6$  cells per plate) were incubated with vehicle (Veh),  $10^{-8}$  mol/L estradiol (E2),  $10^{-7}$  mol/L 4-hydroxytamoxifen (Tam), or 10  $\mu$ mol/L MSA alone, or coincubated with estradiol and MSA or 4-hydroxytamoxifen and MSA for 2 h. ER $\beta$  mRNA was measured by real-time RT-PCR as described in Materials and Methods. Expression was normalized to GAPDH. Columns, means; bars,  $\pm$ SD.

treatment with 2.5  $\mu$ mol/L MSA for 48 hours, whereas MCF-7-LCC2 cells did not show a significant decrease in cell growth until 72 hours of incubation with MSA (Fig. 6D-F). In addition, 1  $\mu$ mol/L MSA reduced growth of MDA-MB-231, MDA-MB-468, and MCF7-H2 $\Delta$ 16 at 72 hours (Fig. 6E and Fig. 7A), whereas the same concentration did not affect growth of MCF-7, MCF-7-LCC2, HEC1A, and Ishikawa cells (data not shown). Currently, the cell-specific sensitivity of MSA is under investigation.

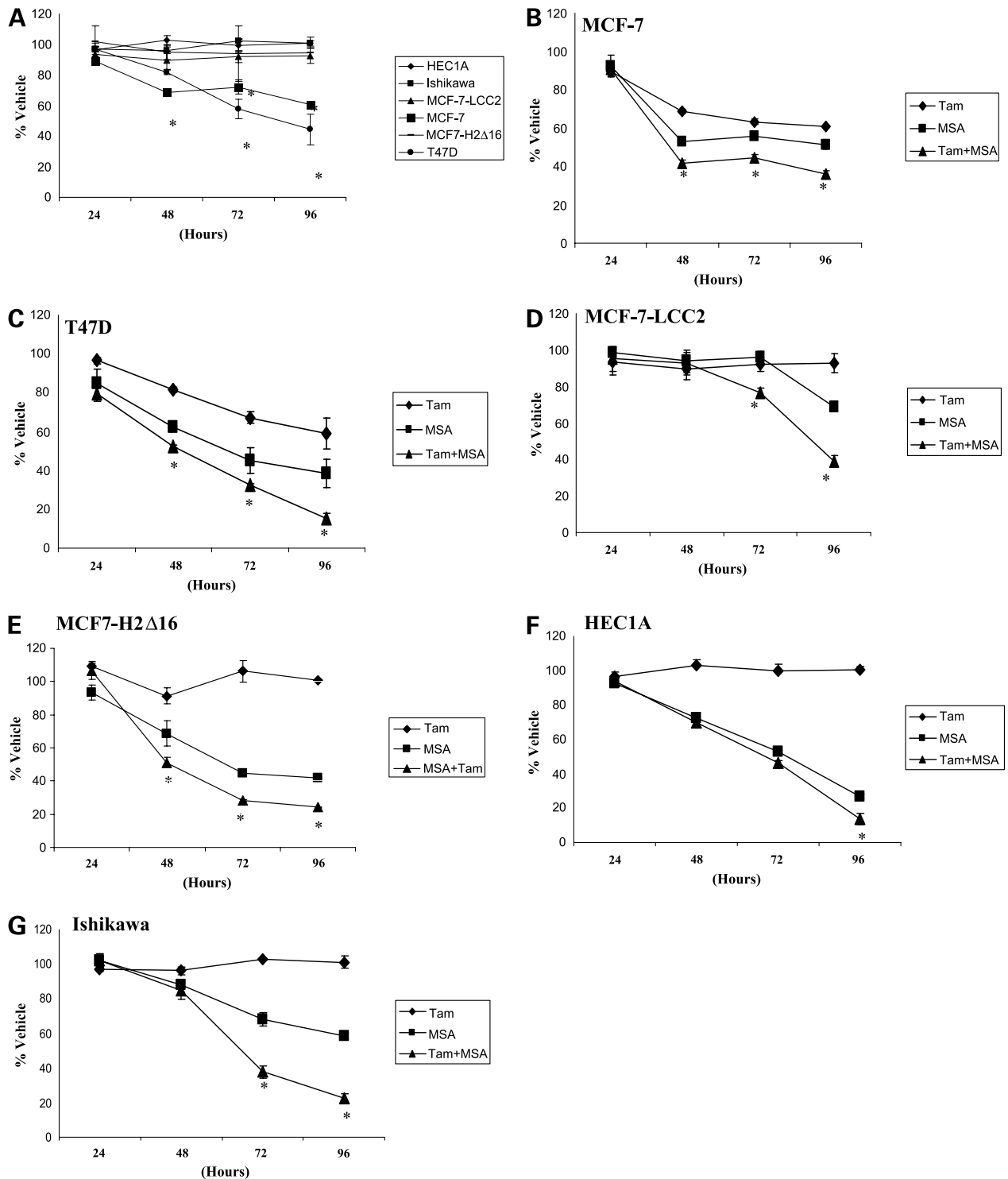
A review of the literature from cancer cell lines and *in vivo* tumors reveals that MSA and tamoxifen exhibit similarities in growth inhibitory mechanisms. Both agents induce G<sub>1</sub> arrest that is associated with a similar profile of changes in cell cycle regulatory proteins (19, 30–32). Both agents induce a dose-dependent apoptosis that is p53 independent and may involve activation of the same caspases as well as reduction in bcl-2 (14, 15, 33–35). This suggests that the added efficacy observed with the combination of selenium with tamoxifen is the result of more pronounced perturbations in several common regulatory proteins resulting in elevated apoptosis and reduced proliferation when compared with effects of either agent alone.

As we have previously shown, low concentrations of MSA (1–2.5  $\mu$ mol/L) had no effect on ER $\alpha$  mRNA and protein expression while still capable of inhibiting estradiol-dependent gene expression.<sup>4</sup> Therefore, at low MSA concentrations, disruption of ER signaling occurred via mechanisms independent of ER $\alpha$  depletion suggesting that MSA also affected ER $\alpha$  function. Dong et al. (35) showed that MSA regulates expression of several proteins known to

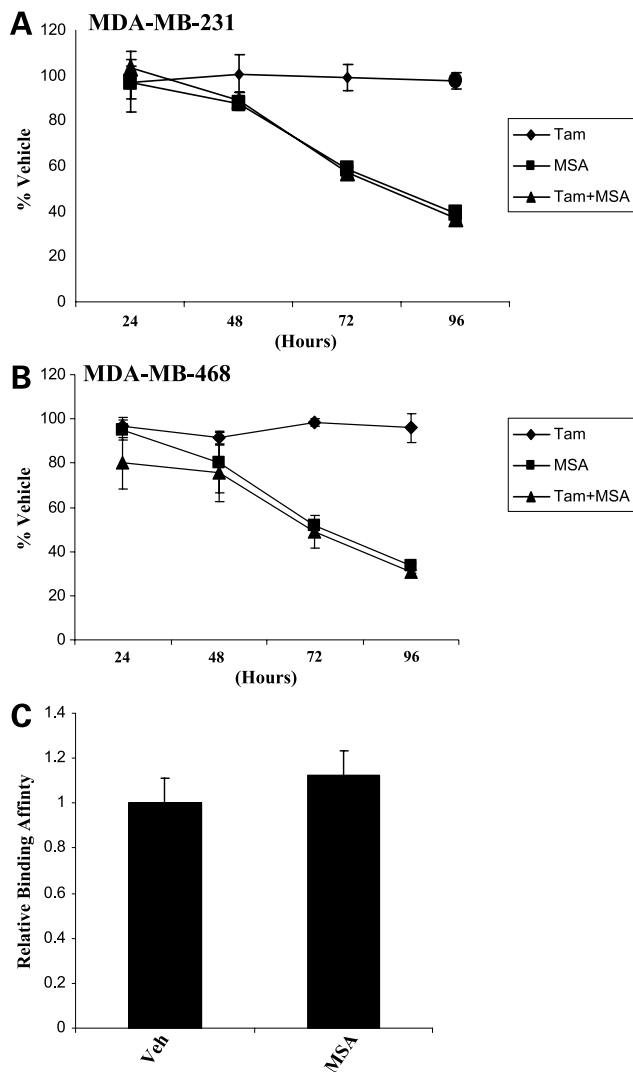
be important mediators of ER $\alpha$  action. In this study, it was shown that MSA decreased cyclin D1 levels in premalignant human breast cancer cells. In addition to its functions as a cell cycle regulator, cyclin D1 is also an ER $\alpha$  coactivator (36) and its overexpression is correlated with tamoxifen resistance in ER $\alpha$ -positive postmenopausal breast cancer (37, 38). Exogenous expression of cyclin D1 in tamoxifen-sensitive breast cancer cells reverses the growth inhibitory properties of tamoxifen (39). In addition to cyclin D1, other coactivators are important in the mechanism of tamoxifen resistance. Overexpression of amplified in breast cancer-1 (SRC-3/RAC-3/ACTR) in patients receiving tamoxifen was correlated with tamoxifen resistance (40). Elevated steroid receptor coactivator-1 (NcoA-1) but not transcriptional intermediary factor-2 (SRC-2/NcoA-2/GRIP1) or amplified in breast cancer-1 was correlated with tamoxifen agonist activity in Ishikawa cells (41). Corepressor levels have been associated with tamoxifen resistance. A decrease in nuclear corepressor levels have been associated with a shorter relapse-free survival in tamoxifen-treated patients suggesting that nuclear corepressor may be a good independent prognostic marker of tamoxifen resistance (42). Dong et al. (35) reported that MSA reduced AKT levels, which is interesting in light of several reports showing increased AKT activity in tamoxifen-resistant breast cancer cells (43, 44). Future studies will elucidate the molecular mechanisms underlying the ability of MSA to restore tamoxifen sensitivity in resistant cells.

Although clinical trials with selenium are currently limited to chemoprevention, recent evidence now strongly





**Figure 6.** MSA increases the growth inhibitory properties of 4-hydroxytamoxifen. **A**, growth inhibitory effects of 4-hydroxytamoxifen as measured by MTT assay. MCF-7, T47D, MCF-7-LCC2, MCF-7-H2Δ16, HEC1A, and Ishikawa cells ( $2 \times 10^4$  cells per well) were incubated with vehicle or  $10^{-7}$  mol/L 4-hydroxytamoxifen for 24, 48, 72, and 96 h. *Columns*, means; *bars*,  $\pm$ SD. Vehicle-incubated samples are set as 100%. \*,  $P < 0.05$ , compared with vehicle. **B**, MCF-7, **(C)** T47D, **(D)** MCF-7-LCC2, **(F)** HEC1A, and **(G)** Ishikawa cells ( $2 \times 10^4$  cells per well) were incubated with vehicle,  $10^{-7}$  mol/L 4-hydroxytamoxifen (*Tam*), or  $2.5 \mu\text{mol/L}$  MSA or coincubated with 4-hydroxytamoxifen and MSA for 24, 48, 72, and 96 h and **(E)** MCF7-H2Δ16 cells were incubated with vehicle,  $10^{-7}$  mol/L 4-hydroxytamoxifen (*Tam*) or  $1 \mu\text{mol/L}$  MSA or coincubated with 4-hydroxytamoxifen and MSA for 24, 48, 72, and 96 h. MTT assays were done at 24, 48, 72, and 96 h posttreatment. Vehicle-incubated samples are set as 100%. \*,  $P < 0.05$ , compared with 4-hydroxytamoxifen- or MSA-incubated samples alone.



**Figure 7.** Tamoxifen and MSA do not synergize for growth inhibition in ER $\alpha$ -negative cell lines. **A**, MDA-MB-231 and **B** MDA-MB-468 cells ( $2 \times 10^4$  cells per well) were incubated with vehicle,  $10^{-7}$  mol/L 4-hydroxytamoxifen (*Tam*), or 1  $\mu$ mol/L MSA, or coincubated with 4-hydroxytamoxifen and MSA for 24, 48, 72, and 96 h, and MTT assays were done. Vehicle incubated samples are set as 100%. **C**, MSA has no effect on ER ligand binding. MCF-7 cells ( $4 \times 10^5$  cells per well) were incubated with vehicle (*Veh*) or 10  $\mu$ mol/L MSA for 1 h. Following 1 h of incubation, 10 nmol/L [ $^3$ H] estradiol in the presence or absence of 500-fold excess cold estradiol was added to each well and incubated for 1 h. Relative binding affinity was measured as described in Materials and Methods, and vehicle-incubated samples were set as 1.

shows the potential of using selenium in a new way, as a novel therapy for overt cancer through combination with well-established chemotherapeutic and hormonal agents. Several studies have shown growth inhibition of established tumors by selenium in *in vivo* models (45–49). However, these studies used inorganic selenium compounds that are genotoxic and no longer used for selenium chemoprevention. More recently, Cao et al. showed a synergistic interaction of organic selenium compounds with the topoisomerase 1 poison irinotecan (50). Xenograft

mice bearing squamous cell carcinomas of the head/neck and colon were given selenium in the form of methylselenocysteine and seleno-L-methionine orally 7 days before i.v. injection of irinotecan. Combination treatment of irinotecan + selenium decreased the toxicity of the chemotherapeutic agent and increased the cure rate of the tumor-bearing mice inoculated with cancer cells sensitive and resistant to irinotecan (50). The present study provides a compelling rationale to explore therapeutic regimens combining tamoxifen with organic selenium compounds for hormone-dependent breast cancer.

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