Selenium sensitizes MCF-7 breast cancer cells to doxorubicin-induced apoptosis through modulation of phospho-Akt and its downstream substrates

Song Li,1 Yunfei Zhou,2 Ruiwen Wang,2 Haitao Zhang,1 Yan Dong,1 and Clement Ip1

Departments of 1Cancer Chemoprevention and 2Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

Abstract

Doxorubicin is an effective drug against breast cancer. However, the favorable therapeutic response to doxorubicin is often associated with severe toxicity. The present research was aimed at developing a strategy of increasing doxorubicin sensitivity so that lower doses may be used without compromising efficacy. The MCF-7 human breast cancer cell line currently in use in our laboratory did not respond to doxorubicin cell killing during a 24-h treatment period. By combining doxorubicin with selenium, we were successful in producing a brisk enhancement of apoptosis. We examined the effects of these two agents on Akt activation and found that selenium was capable of depressing doxorubicin-induced Akt phosphorylation. Several lines of evidence converged to support the notion that this effect is important in mediating the synergy between selenium and doxorubicin. Selenium was no longer able to sensitize cells to doxorubicin under a condition in which Akt was constitutively activated. Increased Akt phosphorylation following treatment with doxorubicin was accompanied by increased phosphorylation of glycogen synthase kinase 3β (GSK3β) and FOXO3A, which are substrates of Akt (both GSK3β and FOXO3A lose their proapoptotic activities when they are phosphorylated). Selenium reduced the abundance of phospho-GSK3β induced by doxorubicin, whereas chemical inhibition of GSK3β activity muted the apoptotic response to the selenium/doxorubicin combination. Additional experiments showed that selenium increased the transactivation activity of FOXO3A, as evidenced by a reporter gene assay, as well as by the elevated expression of Bim (a target gene of FOXO3A). The functional significance of Bim was confirmed by the observation that RNA interference of Bim markedly reduced the potency of selenium/doxorubicin to induce apoptosis. [Mol Cancer Ther 2007;6(3):1031–8]

Introduction

A major challenge facing chemotherapy of solid tumors is the limited efficacy and selectivity of cytotoxic drugs. Recent research by Cao et al. (1) at our institute showed that in nude mice carrying either a human head and neck or colon carcinoma xenograft, daily treatment with selenium increased markedly the cure rate of irinotecan at the maximum tolerable dose of 100 mg/kg/wk × 4. Without selenium, the cure rate was ~25% in the irinotecan-sensitive tumors. With selenium, 100% cure rate was achieved. Selenium by itself produced no significant changes in the growth of the tumor. The above dose schedule of irinotecan was totally ineffective against the irinotecan-resistant tumors (0% cure rate). A high dose of irinotecan (300 mg/kg/wk × 4) was needed to reach >50% cure rate of these resistant tumors, but only in the presence of selenium. The use of such a high dose of irinotecan was made possible due to the selective protection of normal tissues by selenium. Otherwise, this high dose of irinotecan would have caused 100% mortality. Host protection by selenium against drug toxicity was similarly observed with 5-fluorouracil, oxaliplatin, paclitaxel, and doxorubicin.

Doxorubicin and other anthracycline drugs are widely used in the treatment of breast cancer. The benefits in response rate and overall survival, however, are often associated with myelosuppression and cardiomyopathies (2). Thus, it is desirable to develop new modalities that can enhance anthracycline therapeutic efficacy. In the present study, we investigated the potentiation of doxorubicin-induced apoptosis by selenium in the MCF-7 human breast cancer cells. This cell line was derived from the pleural effusion of a patient with metastatic breast cancer (3). Although the MCF-7 model is better recognized for the contribution to antiestrogen therapy research, it is equally suitable for the study of chemosensitization because many of the survival and death signaling pathways are well delineated in this cell model (4). The focus of our investigation is on the modulation of Akt and its downstream substrates by doxorubicin and selenium.

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is known to play an important role in drug sensitivity of MCF-7 cells (4). Akt is a kinase; it phosphorylates a variety of substrates including Bad, glycogen synthase kinase 3β (GSK3β), and FOXO transcription factors. These effector molecules in turn mediate the survival signal of Akt. Bad is
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a proapoptotic Bcl-2 family protein. Once phosphorylated, Bim binds to the 14-3-3 protein; this interaction prevents Bim from translocating to the mitochondria (5). GSK3β is also a proapoptotic mediator due to its involvement in increasing mitochondrial permeability (6, 7). When GSK3β is phosphorylated by Akt, it becomes inactive. FOXO and resuspended in PBS buffer. An aliquot of the cell suspension was mixed with an equal volume of a 0.4% trypan blue solution. Each cell sample was immediately transferred to a hemacytometer for counting in triplicates. Stained (dead) and unstained (viable) cells were counted with an inverted microscope under ×100 magnification.

Measurement of Apoptosis by DNA Fragmentation ELISA

Apoptosis was measured by using the Cell Death ELISA Plus kit (Roche), which quantitatively detects apoptotic nucleosomes. Briefly, cells were seeded onto 24-well plates in DMEM at a density of 20,000 per well and allowed to attach for 24 h. They were then treated with MSA, doxorubicin, or the combination. After a period of 24 h, histone-associated DNA fragments were quantified according to the manufacturer’s instructions.

Western Blot Analysis

Cells were harvested and lysed by 1× lysis buffer (Cell Signaling Technology) containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), 50 mmol/L NaF, and 1 tablet/7 mL of Mini Complete Protease Inhibitor (Roche). Protein concentration of the lysate was determined by using the Bicinchoninic Acid Protein Assay kit from Pierce Biotechnology (Rockford, IL). In preparing for SDS-PAGE, the cell lysate was mixed with 1/3 volume of SDS sample buffer [200 mmol/L Tris-HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol, 60 μL/mL of β-mercaptoethanol] and heated at 100°C for 10 min. Protein bands were visualized by the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology) or the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Construction of Constitutively Activated Akt Plasmid and Transfection

The constitutively activated Akt construct was prepared by incorporating the Lck myristoylation/palmitylation signal (MCWCSS-NPPEDD) to the NH2 terminus of Akt (21), thus allowing Akt to localize to the plasma membrane. MCF-7 cells were seeded at 50% confluence onto six-well plates in DMEM at a density of 20,000 per well and allowed to attach for 24 h. They were then treated with MSA, doxorubicin, or the combination. After a period of 24 h, histone-associated DNA fragments were quantified according to the manufacturer’s instructions.

Transfection of p3xIRS-luc Construct and Luciferase Assay

This construct has three tandem repeats of a FOXO binding element, the insulin-responsive sequence (IRS), inserted upstream of the luciferase reporter gene (22). It is widely used as an indicator of FOXO transcriptional activity. The construct was kindly provided by Dr. Eric D. Tang (University of Michigan, Ann Arbor, MI). MCF-7 cells seeded onto 10-cm plates were transfected with 5 μg of the promoter-luciferase reporter plasmid DNA with the use of Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). Following transfection, the cells were trypsinized, repleted onto six-well plates, and allowed to attach overnight before the addition of MSA to the culture medium. At 24 h, the...
cells were lysed with 1× Passive Lysis Buffer (Promega, Madison, WI). The luciferase activity was measured by using the Luciferase Assay System from Promega and was normalized to the protein concentration in the cell lysate. The transfection experiment was done in triplicate wells and repeated at least four times.

**Bim Small Interfering RNA Transfection**

The SignalSilence Bim small interfering RNA (siRNA) kit was purchased from Cell Signaling Technology. MCF-7 cells were seeded onto 12-well plates at 50% confluence. On day 2, the medium was removed and replaced with 500 μL of fresh serum-containing medium. A 2-μL aliquot of transfection reagent was added to 100 μL of serum-free medium in a sterile microfuge tube, followed by the addition of a 6-μL aliquot of 10 μmol/L stock siRNA to yield a final concentration of 100 nmol/L at transfection. The mixture was incubated for 5 min at room temperature and was then added all at once to the well containing the cultured cells. On day 3, the medium was refreshed and the cells were treated with or without the MSA/doxorubicin combination. On day 4, the cells were harvested and cell lysates were prepared for Western blot analysis.

**Results**

**Synergy between MSA and Doxorubicin in Causing Cell Death**

Although flow cytometry analysis of Annexin V staining is a well-accepted assay for detecting apoptotic cell death, this method is not suitable for cells treated with doxorubicin. The reason is because doxorubicin emits a very strong, broad-band fluorescence that interferes with the assay. In view of the above problem, we initially used trypan blue staining to quantify cell death caused by MSA, doxorubicin, or the combination. Dead cells take up the dye whereas viable cells do not. By counting 1,000 cells each time and repeating the experiment four times, the percentage of dead cells was calculated. As shown in Fig. 1A, treatment with 2.5 or 5 μmol/L MSA over a 24-h period did not increase cell death compared with the untreated control. Likewise, doxorubicin at 200 or 400 nmol/L produced no or minimal increase in cell killing. On the other hand, a combination of MSA and doxorubicin was definitely more potent, especially when 5 μmol/L MSA was added together with 200 or 400 nmol/L doxorubicin.

Trypan blue staining quantifies nonviable cells. It makes no distinction of cell death by apoptosis. To clarify this issue, we used the Roche DNA fragmentation Cell Death Detection ELISA method to study the synergy between MSA and doxorubicin. This method is highly specific for quantifying apoptotic cell death. Cells were treated for 24 h with 5 μmol/L MSA alone, 400 nmol/L doxorubicin alone, or the combination. An advantage of the ELISA method is that it is quantitative. The results in Fig. 1B show that MSA caused only a tiny increase in cell death whereas the doxorubicin effect was a bit more visible. The response to the combination, however, was decidedly more robust.

It is clear that MSA and doxorubicin work cooperatively to commit cells to apoptosis.

A hallmark of caspase-dependent apoptosis is the proteolytic cleavage of PARP, an enzyme involved in DNA damage repair and maintenance of genome stability. PARP cleavage is widely used as a sensitive indicator of caspase-mediated apoptotic cell death. As shown in Fig. 1C, PARP cleavage was not detectable when cells were treated with MSA alone (at 2.5 or 5 μmol/L) or doxorubicin alone (at 200 or 400 nmol/L). In contrast, the cleaved PARP band was much more noticeable with the combination treatment. The magnitude of the increase was proportional to the dosage of each drug in the combination. In other words, the strongest band was observed with 5 μmol/L MSA/400 nmol/L doxorubicin and the weakest with 2.5 μmol/L MSA/200 nmol/L doxorubicin.

In summary, the synergy between MSA and doxorubicin in causing cell death was consistently observed across three different assays. The MCF-7 cell line currently in use in our laboratory is not particularly sensitive to apoptosis induction by either MSA or doxorubicin, at least not with the doses of each drug used here and in the time frame as
described in the above experiments. The drug dose issue will be revisited later. With the above information, it is not possible to distinguish whether MSA is sensitizing cells to doxorubicin or vice versa. This question can only be answered at the molecular level.

**Evidence of Doxorubicin Sensitivity Based on Molecular Changes**

Doxorubicin induction of apoptosis is known to be dependent on p53. To elucidate whether the failure to observe apoptosis in our study might be due to some defect in the p53 mechanism, we examined the expression of p53 as well as Bax (a target gene of p53) in MCF-7 cells treated with 200 or 400 nmol/L doxorubicin. The results are shown in Fig. 2. We found that doxorubicin increased the expression of both proteins in a dose-dependent manner, suggesting that p53 signaling is intact in our cells. Is it possible that some survival pathway is activated to offset the effect of p53? This could explain the disconnect between the p53 data and the apoptosis data.

**Up-regulation of Akt Phosphorylation by Doxorubicin**

As alluded to in Introduction, Akt activation is a key determinant in defining sensitivity to chemotherapeutic drugs. To confirm that this is also true in our model, we treated MCF-7 cells with 200 or 400 nmol/L doxorubicin for 24 h and collected the cell lysate for Western blot analysis of phospho-Akt. The results in Fig. 3A show that doxorubicin increased Akt Ser473 phosphorylation in a dose-dependent manner without affecting total Akt expression. We also investigated the functional relevance of Akt activation by using a chemical inhibitor (LY294002) to block the activity of PI3K. The presence of the inhibitor is expected to overcome the barrier to apoptosis induction if the PI3K/Akt pathway is of consequence. We studied PARP cleavage as a marker of apoptosis. The results are shown in Fig. 3B. There was indisputable evidence of cleaved PARP when cells were treated with doxorubicin + LY294002. The observation suggests that a down-regulation of PI3K/Akt signaling is able to restore sensitivity to doxorubicin. Negative controls were provided by doxorubicin alone or LY294002 alone. Although blocking Akt expression by siRNA would offer a more direct proof of the role of Akt, we decided to use a PI3K inhibitor for the above experiment. The reason is that the up-regulation of phospho-Akt by doxorubicin might be PI3K dependent, as has previously been suggested by Li et al. (14).

**Functional Significance of MSA Reversal of Akt Activation by Doxorubicin**

The objectives of the next series of experiments were to investigate (a) the efficacy of MSA in repressing basal as well as doxorubicin-induced phospho-Akt levels and (b) whether the overexpression of constitutively activated Akt would diminish doxorubicin sensitization by MSA. In the first experiment, cells were treated with 5 μmol/L MSA alone for 12 or 24 h. We found that MSA decreased phospho-Akt at both time points (Fig. 4A). The control band was considerably stronger in this experiment than in the previous experiment because the blots in Fig. 4A were exposed to the film for a longer period of time. Otherwise, it would have been difficult to detect the ability of MSA to reduce basal phospho-Akt level. In the second experiment, cells were treated with either 5 μmol/L MSA or 400 nmol/L doxorubicin, or the combination. MSA clearly repressed doxorubicin-induced phospho-Akt at both 12 and 24 h (Fig. 4B). The third experiment was designed to study whether the down-regulation of phospho-Akt by MSA contributed to doxorubicin sensitization. We transfected cells with either the constitutively activated Akt or the empty vector, and then treated both sets of cells with MSA + doxorubicin. The cells were harvested after 24 h for analysis of PARP cleavage and DNA fragmentation. The overexpression of Akt was confirmed by Western blot (Fig. 4C). In the absence of drugs, very little cleaved PARP (Fig. 4C) and DNA fragmentation (Fig. 4D) were observed in either the empty vector–transfected cells or the Akt-transfected cells. However, the transfection of constitutively activated Akt markedly muted the ability of the drugs

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**Figure 2.** Western blot analysis of p53 and Bax induction by doxorubicin.

**Figure 3.** Role of phospho-Akt up-regulation by doxorubicin. **A,** Western blot analysis of phospho-Akt and total Akt in cells treated with doxorubicin for 24 h. **B,** enhancement of PARP cleavage by doxorubicin in the presence of LY294002, an inhibitor of PI3K.

**Table 1:**

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to increase PARP cleavage and DNA fragmentation, suggesting that the down-regulation of Akt activation by MSA is responsible, at least in part, for doxorubicin sensitization.

**GSK3β and Doxorubicin Sensitization by MSA**

GSK3β is a substrate of Akt. The phosphorylation changes of GSK3β should follow a pattern similar to that of Akt in the presence of doxorubicin or doxorubicin + MSA. As shown in Fig. 5A, doxorubicin increased phospho-GSK3β, whereas MSA reversed this effect. Total GSK3β was not significantly affected by either drug. Phosphorylation of GSK3β inactivates the activity of GSK3β. Because GSK3β is known to produce a proapoptotic response, blocking the activity of GSK3β is expected to dampen apoptosis. We used a specific chemical inhibitor of GSK3β, SB216763, to study its effect on the induction of PARP cleavage by doxorubicin + MSA. The results are shown in Fig. 5B. Consistent with our expectation, adding the GSK3β inhibitor to the culture medium markedly reduced PARP cleavage in cells treated with the two drugs, suggesting that the reactivation of GSK3β by MSA might contribute to doxorubicin sensitization.

**FOXO3A/Bim and Doxorubicin Sensitization by MSA**

FOXO3A is also a substrate of Akt. Phosphorylation of FOXO3A decreases its transactivation due to the exit of FOXO3A from the nucleus to the cytoplasm. Conversely, reducing phospho-FOXO3A is expected to enhance its transactivation. We first examined the modulation of phospho-FOXO3A by MSA (Fig. 6A). In parallel with the phospho-Akt data, phosphorylation of FOXO3A was lowered by MSA at 12 and 24 h, whereas the total expression of FOXO3A was not affected. To assess the transactivation activity of FOXO3A, we transfected cells with the p3xIRS-luciferase reporter construct, followed by treatment with MSA for 24 h. As shown in Fig. 6B, MSA indeed significantly increased the luciferase activity in the transfected cells. Originally, we had planned to determine whether siRNA knockdown of FOXO3A would reverse doxorubicin sensitization by MSA. We tested several commercial FOXO3A siRNA reagents. None of them was able to decrease FOXO3A to our satisfaction. We had to abandon this approach.

Bim, a proapoptotic Bcl-2 protein, is a target gene of FOXO3A. The expression of Bim was analyzed by Western blot in cells treated with doxorubicin, MSA, or the combination. The 12-h and 24-h time point data are shown in Fig. 7A. Doxorubicin by itself decreased slightly the expression of Bim. MSA, on the other hand, increased markedly Bim expression with or without doxorubicin. Could Bim induction by MSA be responsible for doxorubicin sensitization? To address this question, RNA interference of Bim was carried out to study how Bim knockdown might affect apoptosis induction by doxorubicin + MSA. A scrambled sequence was used as the negative control. Bim siRNA successfully reduced the high level of Bim induced by MSA (Fig. 7B) and greatly compromised PARP cleavage and caspase-9 activation caused by doxorubicin and MSA (Fig. 7C). The results suggest that Bim might be an important mediator in doxorubicin sensitization by MSA.

In summary, although we were unable to pinpoint the functional significance of FOXO3A itself, we achieved the goal of evaluating the contribution of a FOXO3A target gene. In retracing our steps back to Akt, we believe that the

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**Figure 4.** Down-regulation of phospho-Akt by MSA and doxorubicin sensitization. A, repression of phospho-Akt by 5 μmol/L MSA. B, modulation of phospho-Akt by 5 μmol/L MSA, 400 nmol/L doxorubicin, or the combination. C, PARP cleavage by MSA/doxorubicin in cells transfected with the constitutively activated Akt (designated as Akt construct plus). Cells transfected with the empty vector (designated as Akt construct minus) served as the negative control. D, apoptosis induction (DNA fragmentation ELISA) by MSA/doxorubicin in cells transfected with the constitutively activated Akt. *, P < 0.05, significantly different than the value obtained from the Akt construct minus sample.
following sequence of events take place in our cell model:
MSA reversal of Akt activation by doxorubicin
restored transactivation of FOXO3A
up-regulation of Bim
sensitization to doxorubicin.

Discussion
We have characterized selenium repression of doxorubicin-induced Akt activation as a viable approach to sensitize cancer cells to doxorubicin. Our conclusion is supported by the following arguments. First, overexpressing constitutively activated Akt blocked the ability of MSA/doxorubicin to enhance apoptosis. Second, doxorubicin increased the phosphorylation of two Akt substrates, viz. GSK3β and FOXO3A, whereas MSA reversed this effect. When phosphorylated, both GSK3β and FOXO3A lose their proapoptotic activities. Thus, MSA helps to maintain the function of these two proteins. Third, chemical inhibition of GSK3β activity negated the effect of doxorubicin sensitization by MSA. Fourth, MSA increased FOXO3A transactivation. The functional significance of Bim, a FOXO3A target gene, was confirmed by the observation that RNA interference of Bim markedly reduced the potency of doxorubicin/MSA to induce apoptosis. Akt phosphorylates a host of substrates in addition to GSK3β and FOXO3A. Our study is not meant to imply that GSK3β and FOXO3A are more important than the rest. We picked two representative Akt substrates to illustrate the potential of modulating Akt signaling as a strategy to increase drug sensitivity.

How does MSA decrease Akt phosphorylation? Our previous work in PC-3 human prostate cancer cells showed that MSA inhibits the activity of PI3K, thereby disrupting the recruitment of phosphoinositide-dependent kinase 1 and Akt to the plasma membrane (19). We also found that MSA may lessen the phosphorylation of Akt at the Ser473 site via the phosphatase action of calcineurin (19). The activity of calcineurin is dependent on calcium, and MSA enhances calcium release from the endoplasmic reticulum. Presently, we are also investigating the signals emanating from endoplasmic reticulum stress as molecular switches turned on by MSA in facilitating a commitment to apoptosis (23, 24). The mechanisms of endoplasmic reticulum stress–associated apoptosis include a direct release of caspases from the endoplasmic reticulum, as well as an indirect activation of both the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways. These pleiotrophic effects of selenium make it unique as a chemotherapeutic modulator. The synergy of doxorubicin and MSA in increasing apoptosis is not special to the MCF-7 cells. We have additional data showing that MDA-MB-231 breast cancer cells (estrogen receptor negative, p53 mutant) respond similarly to the drug combination. Thus, it is clear that MSA has different ways to enhance drug sensitivity in the presence or absence of a functional p53.

This discussion will not be complete without a few comments about the doses of doxorubicin and MSA used in our experiments. The pharmacology of doxorubicin administered by a 96-h continuous intravenous infusion method in humans has been studied (25). The steady-state concentration of doxorubicin in the plasma during this period is in the order of 100 nmol/L. In our experiments, cells were treated with 200 or 400 nmol/L doxorubicin for 12 or 24 h. Thus, the total dose intensity (i.e., concentration integrated over time) in our in vitro model is clinically relevant. A recent phase I study at our institute showed that daily administration of selenomethionine to colon cancer patients (also treated with irinotecan) could achieve
plasma selenium concentration in excess of 25 μmol/L without any symptom of toxicity (26). Although specification of selenium metabolites in biological samples is not possible at the present time, it is reasonable to assume that a significant portion of selenium in tissues is in a form that has comparable activity to that of MSA. As can be seen in our study, a level of 5 μmol/L MSA is sufficient to downregulate Akt signaling in the normal tissues if it were to promote their survival. We have reasons to believe that selenium has divergent effects on molecular changes in normal versus cancer tissues and that the qualitative response to selenium is very much dependent on metabolic, oxidative, and other forms of stress, which are inherently different between normal and cancer tissues. This should be a fruitful area of research in the future.

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