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Award Number: DAMD17-02-1-0007

TITLE: Methylselenium and Prostate Cancer Apoptosis

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REPORT DATE: February 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-02-2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 4 JAN 2005 - 3 JAN 2006	
4. TITLE AND SUBTITLE Methylselenium and Prostate Cancer Apoptosis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-02-1-0007	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Junxuan Lu, Ph.D. E-mail: jlu@hi.umn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Minnesota Minneapolis, MN 55455-2070				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of prostate cancer (PCa) cells by methyl selenium (Se)/selenol. We hypothesized that methyl selenium inhibits PI ₃ K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. The specific aims are to delineate the caspase-mediated execution pathways of apoptosis (Objective 1) and to critically test the role of PI ₃ K-AKT survival pathway in apoptosis signaling (Objective 2) induced by methyl Se/selenol. We have continued experiments pertinent to these two objectives and have pursued a novel lead for methyl Se as a chemosensitizer for cancer therapeutic drugs in androgen independent PCa cells, which are very resistant to conventional chemotherapy. Specifically, we established the MSeA, but not selenite, enhanced apoptosis induced by 3 clinically relevant drugs, taxol, etoposide and CPT-11. We established that the enhancement was mediated by increased activation of caspases. We established a crucial role of the JNK pathway in mediating death signals from all three drugs. Key results were published in Clinical Cancer Res 2005. In the next year, we plan to investigate the role and interplay with PI3K/AKT and ERK regulation of the chemosensitization by MSeA. These mechanistic investigations will lay ground work for future validation studies in vivo and translation from the bench to the bedside.					
15. SUBJECT TERMS Selenium, methylselenol, prostate cancer chemoprevention, apoptosis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 24	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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1. Introduction

The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of PCa cells by methyl Se/selenol. We hypothesized that methyl Se/selenol inhibits PI₃K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. The specific aims are to delineate the caspase-mediated execution pathways of apoptosis (Objective 1) and to critically test the role of PI₃K-AKT survival pathway in apoptosis signaling (Objective 2) induced by methyl Se/selenol.

STATEMENT OF WORK

Objective 1: To define the generalizability and specific features of the caspase-mediated apoptosis execution pathway(s) triggered by methyl Se/selenol pool in PCa cell lines of different androgen dependence and malignancy states.

Test essentiality of caspases and cell detachment for apoptosis induction by methyl Se/selenol in LNCaP, PC-3 and PrEC cells. Correlate PTEN and AKT status with apoptosis sensitivity to methyl Se/selenol.

Delineate caspase cascades invoked by methyl Se/selenol with caspase inhibitors. Test role of mitochondria for apoptosis signaling induced by methyl Se/selenol.

Objective 2: To critically test the mediator role of AKT inhibition as an upstream signaling pathway for methyl Se/selenol-induced PCa apoptosis

Test effects of PI3K inhibitors LY294001 and wortmannin alone or in combination with methyl Se/selenol on apoptosis signaling and execution in DU145 and other PCA cells.

Restore AKT activity in stable transfectants of a constitutively-active AKT (AKT) in DU145 cells. Select for 3 clones expressing low, moderate and high level of the transgene.*

Compare their apoptosis responses to methyl Se/selenol with vector transfectants (control) in dose response and time course experiments.

2. Key accomplishments

2.1 We have in the current reporting period continued experiments related to both approved objectives above. Key results have been published in ***Carcinogenesis*** 2005 (See reprint, **Appendix 1**). Detailed discussion of these accomplishments has been described in the revised and updated progress report for 2004 (submitted in June 2005).

2.2 During the execution of the experiments related to these two objectives, we have discovered and pursued a novel activity of methylselenium for specific potentiation of apoptosis potency of several chemotherapeutic drugs as a **chemosensitizer**. This has significant implications for improving the chemotherapy of androgen-independent PCa by selenium. The results have been published in Clin Cancer Res, 2005 and highlighted below.

Purpose: To test whether and how selenium enhances the apoptosis potency of selected chemotherapeutic drugs in prostate cancer (PCA) cells.

Experimental Design: DU145 and PC3 human androgen-independent PCA cells were exposed to minimal apoptotic doses of selenium (compared MSeA with Selenite) and/or the topoisomerase I inhibitor 7-ethyl-10-hydroxycamptothecin (SN38), the topoisomerase II inhibitor etoposide or the microtubule inhibitor paclitaxel/taxol. Apoptosis was measured by ELISA for histone-associated DNA fragments, by flow cytometric analysis of sub-G1 fraction, and by immunoblot analysis of cleaved poly(ADP-ribose)polymerase (PARP). Pharmacologic inhibitors were used to manipulate caspases and c-Jun-N-terminal kinases (JNK).

Results: The methylselenol precursor methylseleninic acid (MSeA) increased the apoptosis potency of SN38, etoposide or paclitaxel by several folds higher than the expected sum of the apoptosis induced by MSeA and each drug alone (**See reprint 2, Figure 1**). The combination treatment did not further enhance JNK1/2 phosphorylation that was induced by each drug in DU145 cells (**Figure 3**). The JNK inhibitor SP600125 substantially decreased the activation of caspases and apoptosis induced by MSeA combination with SN38 (**Fig. 4**) or etoposide (**Fig. 5A**) and completely blocked these events induced by MSeA/paclitaxel (**Fig. 5C**). The caspase-8 inhibitor zIETDfmk completely abolished apoptosis and caspase-9 and caspase-3 cleavage, whereas the caspase-9 inhibitor zLEHDfmk significantly decreased caspase-3 cleavage and apoptosis but had no effect on caspase-8 cleavage (**Figure 5**). None of these caspase inhibitors abolished JNK1/2 phosphorylation. A JNK-independent suppression of survivin by SN38 and etoposide, but not by paclitaxel, was also observed (**Figure 6**). In contrast to MSeA, selenite did not show any enhancing effect on the apoptosis induced by these drugs (**Figure 1**).

These results indicate that MSeA enhanced apoptosis induced by cancer therapeutic drugs in androgen-independent PCA cells. In DU145 cells, the enhancing effect was primarily through interactions between MSeA and JNK-dependent targets to amplify the caspase-8-initiated activation cascades. The results suggest a novel use of methyl selenium for improving the chemotherapy of PCA through increasing caspase activities.

Hormone refractory PCa are not sensitive to most chemotherapeutic drugs in patients. If methylselenium can indeed make these cancers more responding to existing drugs, then we can anticipate to improve clinical care of patients.

3. Reportable outcomes

Peer reviewed Publications

Hongbo Hu, Cheng Jiang, Guangxun Li and Junxuan Lü: PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 2005 26(8):1374-1381.

Hongbo Hu, Cheng Jiang, Clement Ip, Youcef M. Rustum, and Junxuan Lü. Methylseleninic acid potentiates apoptosis induced by chemotherapeutic drugs in androgen independent prostate cancer cells. *Clin Cancer Res.* 2005 11:2379-2388.

Lu J, Jiang C. Selenium and cancer chemoprevention: hypotheses integrating the actions of selenoproteins and selenium metabolites in epithelial and non-epithelial target cells. *Antioxid Redox Signal.* 2005 Nov-Dec; 7(11-12):1715-27. Invited review.

4. Conclusions

Work conducted during this current reporting period has further strengthened the specific role of AKT pathway in modulating methyl Se induced caspase-mediated death, leading to the inference that this survival protein kinase may be a key determinant of chemopreventive and therapeutic efficacy of methyl Se.

Furthermore, we established caspase-activation as an important pathway for methylselenium to potentiate chemotherapeutic drug efficacy in androgen independent PCa cells. This chemosensitizer activity of selenium is expected to improve quality of life of cancer patients because of increased efficacy and reduced dosage needed.

In the next year, we would like to define the early signaling mechanisms induced by methylselenium for apoptosis and how and through what targets AKT regulates death signaling. We would also like to investigate the role of AKT pathway in the regulating the ability of methylselenium to enhance drug-induced apoptosis. Such mechanistic pursuit will lay the ground work for in vivo validation studies in the future. In vivo validation is a critical step towards the eventual translation of our findings to the bedside.

5. Appendices

1. Hongbo Hu, Cheng Jiang, Guangxun Li and Junxuan Lü: PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 2005 26(8):1374-1381.

2. Hu H, Jiang C, Ip C, Rustum YM, Lu J. Methylseleninic acid potentiates apoptosis induced by chemotherapeutic drugs in androgen-independent prostate cancer cells. *Clin Cancer Res.* 2005 11(6):2379-88.

PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells

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Methylselenol has been implicated as an active metabolite for the anticancer effect of selenium in part through the induction of cancer cell apoptosis. Since inactivation of the AKT/protein kinase B negative regulator gene PTEN (phosphatase and tensin homologue deleted on chromosome 10) is common in prostate cancer (PCa), we compared PTEN wild-type DU145 PCa cells (low basal AKT activity) with PTEN-mutant LNCaP PCa cells (high basal AKT activity) for their apoptosis responses to the methylselenol precursor methylseleninic acid (MSeA) and sodium selenite, an inorganic salt. Our results show that LNCaP cells withstood ~4 times higher doses of MSeA than DU145 cells, although they were slightly more sensitive than the latter to selenite-induced apoptosis. Treatment by MSeA modestly attenuated AKT phosphorylation and increased phospho-ERK1/2 in LNCaP cells. Selenite treatment increased the phosphorylation of p53 Ser15 and both kinases, but the selenite-induced apoptosis was not influenced by chemical inhibitors of either kinase. In contrast, PI3K/AKT inhibitors greatly sensitized LNCaP cells to apoptosis induced by MSeA, accompanied by increased mitochondrial release of cytochrome *c* and multiple caspase activation without changing p53 Ser15 phosphorylation. The apoptosis was further accentuated by extracellular signal regulated kinases 1 and 2 (ERK1/2) inhibition without further increase in cytochrome *c* release. The general caspase inhibitor z-VAD-fmk completely blocked MSeA-induced apoptosis when both kinases were inhibited, whereas a caspase-8 inhibitor exerted a greater protection than did a caspase-9 inhibitor. Transfection of DU145 cells with a constitutively active AKT increased their resistance to MSeA-induced apoptosis. In summary, AKT played an important role in regulating apoptosis sensitivity of LNCaP and DU145 cells to MSeA. An MSeA-induced activation of ERK1/2 in LNCaP cells also contributed to resistance to apoptosis. However, these kinases did not significantly regulate caspase-mediated apoptosis induced by selenite in LNCaP cells. These findings support the differential involvement of these protein kinase pathways in regulating apoptosis induction by different forms of selenium.

Introduction

Despite advances in early detection and treatments, prostate cancer (PCa) continues to be one of the biggest health problems for aging men in USA. Almost 200 000 new cases were projected in 2004 and 30 000 men will succumb to this disease (1) (http://www.cancer.org/docroot/stt/stt_0.asp). Chemoprevention using synthetic or naturally occurring agents that inhibit one or more steps in the natural history of prostate carcinogenesis holds great promise to decrease the morbidity and mortality of PCa (2). The essential trace element selenium has been shown as a promising preventive agent for PCa in a human clinical trial by Clark and co-workers (3,4) and additional trials are being conducted to verify this efficacy (5–7). Mechanistically, induction of apoptosis is believed to be a critical cellular event in PCa chemoprevention and therapy by selenium compounds (8). Methylselenol has been implicated as an active anticancer selenium metabolite (9–13).

We have earlier shown that methylselenol and its synthetic penultimate precursor compound methylseleninic acid (MSeA) induce caspase-mediated apoptosis in DU145 PCa cells (14,15) and have observed that cell death is associated with decreased phosphorylation of AKT/protein kinase B and extracellular signal regulated kinases 1 and 2 (ERK1/2) (14–16). The phosphatidylinositol 3-kinase (PI3K)–AKT pathway has been shown to inhibit apoptosis in most cell types (17,18) and promote angiogenesis (19). In ~50% of prostate tumors, this pathway is constitutively upregulated owing to the deletion of the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) (20–22), which functions as a negative regulator of PI3K in part through lipid phosphatase activity (23). The ERK1/2 pathway is primarily known for mitogenic signaling and for modulating cell survival in most model systems (24,25). Constitutive activation of ERK1/2 has been observed in some cancer types including the DU145 PCa cells (26,27). These findings support the important roles of the PI3K/AKT and ERK1/2 signaling pathways in PCa development. It is of significant interest in terms of targeted therapy or chemoprevention whether the mutational/functional status of these pathways could be molecular regulators or determinants of the potency of selenium for apoptosis induction in PCa cells.

To this end, we have compared the sensitivity of three classic PCa cell lines for the induction of apoptosis by MSeA and selenite, an inorganic salt that we and others have shown to induce DNA single strand breaks (28–30). The androgen-independent DU145 cells possess a wild-type PTEN and low basal AKT activity, and constitutively active ERK1/2 (26,27). The androgen-sensitive LNCaP cells and androgen-independent PC-3 cells are PTEN negative with greater basal AKT activity than the DU145 cells (26,27). We report here that LNCaP and PC-3 cells are more resistant to the induction of apoptosis by MSeA than DU145 cells in a manner proportional to their basal AKT activities. We show that inhibition of AKT

Abbreviations: ERK, extracellular signal regulated kinase; GSK-3, glycogen synthase kinase-3; MSeA, methylseleninic acid; PCa, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

by either a PI3K inhibitor or AKT inhibitors sensitized LNCaP cells to the induction of apoptosis by MSeA, whereas transfection of a constitutively active AKT (CA-AKT) into DU145 cells increased their resistance to MSeA. Furthermore, inhibiting MSeA-induced ERK1/2 activation accentuated apoptosis in LNCaP cells, implicating a unique survival response that further contributed to the resistance phenotype. However, inhibiting these two pathways did not affect selenite-induced apoptosis in LNCaP cells.

As far as caspase mediators are concerned, we focused on caspase-8 and caspase-9 as representatives of two well characterized caspase activation pathways (31,32). The mitochondrial (intrinsic) pathway generally involves the loss of transmembrane potential and the release of cytochrome *c* into the cytosol. Cytosolic cytochrome *c* associates with APAF-1 and procaspase-9 to form a protein complex known as the apoptosome, leading to the cleavage activation of procaspase-9, which in turn cleaves and activates the effectors caspase-3 and caspase-7, and finally to the characteristic proteolysis, e.g. poly(ADP-ribose) polymerase (PARP) cleavage (33) and DNA digestion. The death receptor (extrinsic) pathway involves the engagement of the death receptors, recruits the adapter protein FADD and procaspase-8 to form a complex known as the death inducing signaling complex or DISC. The consequent proximity of procaspase-8 proteins in the DISC allows their autocleavage activation. Caspase-8 can directly activate caspase-3, and caspase-7 to lead to PARP cleavage and DNA oligonucleosomal digestion. Caspase-8 can also activate caspase-9 cascade by cross-talk through cleaving Bid, leading to the translocation of the truncated Bid to the mitochondria where it facilitates the release of cytochrome *c* and the activation of the intrinsic pathway. Our data support AKT and ERK suppression of both extrinsic and intrinsic caspase cascades to attenuate apoptosis sensitivity for MSeA in LNCaP cells without affecting p53 phosphorylation.

Materials and methods

Chemicals and reagents

MSeA ($\text{CH}_3\text{SeO}_2\text{H}$) was synthesized as a precursor for methylselenol for cell culture studies (11,12) and was a generous gift of Dr Howard Ganther, University of Wisconsin. We have shown earlier that methylselenol generated by reacting selenomethionine with recombinant methioninase recapitulated morphological apoptosis and biochemical markers induced by MSeA (15). PI3K inhibitor LY294002, AKT specific inhibitor (Catalog No. 124005) and NL-71-101 (Catalog No. 487940) were purchased from CalBiochem (La Jolla, CA). MEK inhibitor U0126 was purchased from Promega (Madison, WI). Caspase inhibitors (z-VAD-fmk, z-IETD-fmk and z-LEHD-fmk) were purchased from MP-Biomedical (Aurora, OH). Phospho-specific antibodies for AKT (Ser473), ERK1/2 (Thr202/Tyr204) and caspase antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). Mitochondria-free cytosol fractionation kit and cytochrome *c* antibody were purchased from BD Biosciences (Palo Alto, CA).

Cell culture and treatments

DU145, LNCaP and PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LNCaP cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine without antibiotics. DU145 cells were cultured in Minimum Essential Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics. PC-3 cells were cultured in F-12K medium supplemented with 10% FBS and 2 mM L-glutamine without antibiotics. Cells at 50–60% confluence, usually 24–48 h after plating, were given a medium change and treated with selenium. PI3K, AKT or MEK inhibitors were dissolved in dimethylsulfoxide (DMSO) as concentrated stocks. In experiments involving the kinase inhibitors, fresh media containing a desired concentration of each inhibitor were fed to cells for 1 h and then additional treatments were initiated. Caspase inhibitors and MSeA were mixed into

treatment media first and then fed to cells. DMSO (2 $\mu\text{l}/\text{ml}$ or less) was added to groups that did not receive the inhibitors to control the solvent vehicle effects. DMSO at the concentration used did not by itself cause any observable adverse morphological responses.

Apoptosis and caspase assays

After treatments, both adherent and floating cells were collected. A cell death ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN) was used to measure oligonucleosomes released by apoptotic cells. Cleavages of PARP and procaspases were detected by immunoblot as markers of caspase-mediated apoptosis as described previously (14). Caspase activity was measured as described previously with assay kits from R&D Systems (Minneapolis, MN) (34).

Preparation of cytosolic extracts

A Cell Fractionation Kit (Clontech-BD Biosciences, Palo Alto, CA) was used to prepare cytosolic extracts for cytochrome *c* detection. Both adherent and floating cells were collected by centrifugation. The cells were resuspended in 1 ml of ice-cold wash buffer provided by the Kit. After washing, the cell pellets were resuspended in 0.8 ml of ice-cold fractionation buffer and incubated on ice for 10 min. The cells were homogenized in an ice-cold Dounce tissue grinder with 60 passes. After centrifugation at 12 000 *g* for 25 min, the mitochondria-free supernatant was collected for immunoblot analysis of cytochrome *c*.

Stable transfection of DU145 cells with a CA-AKT1

The mouse CA-AKT1 in pUSEamp plasmid was kindly provided by Dr Zigang Dong (Hormel Institute, University of Minnesota). DU145 cells were seeded into 6-well plates until ~60% confluence and were transfected using Lipofectamine 2000 purchased from Invitrogen (Carlsbad, CA). Briefly, 2 μg of plasmid and 10 μl of Lipofectamine 2000 were diluted into 100 μl of serum-free medium, respectively. After 5 min incubation at room temperature, the two solutions were combined with gentle mixing. After incubation for 20 min at room temperature, the mixture was added to each well which contained 0.8 ml serum free medium. After 6 h incubation at 37°C, the transfection medium was replaced with 2 ml of complete medium. After 24 h incubation, the transfected cells were harvested and replated into 100 mm plates and replaced with selection medium containing 800 $\mu\text{g}/\text{ml}$ of G418 the following day. Resistant colonies were selected after ~6 weeks. The AKT activity of each clone was measured as described below.

In vitro AKT kinase assay

In vitro AKT kinase assay was carried out as per the manufacturer's instructions by an AKT Kinase Assay Kit purchased from Cell Signaling Technology (Beverly, MA). Briefly, the cells were harvested and washed twice with phosphate buffered saline, and lysed in ice-cold lysis buffer provided by the kit. Then 200 μg of protein was immunoprecipitated with 2 μg of anti-AKT antibody overnight. After extensive washing, the immunoprecipitates were incubated with 1 μg of glycogen synthase kinase-3 (GSK-3) fusion protein substrate in 50 μl of kinase buffer for 30 min at 30°C. Reactions were terminated by SDS loading buffer. The samples were separated on 12% SDS-PAGE, and the phospho-GSK-3 α/β (Ser219) was detected by immunoblotting.

Statistical analysis

Where appropriate, ANOVA was used to test the significance of the differences among the treatment groups and their relative controls. Statistical significance was determined at the 0.05 or 0.01 level using Student's *t*-test.

Results

LNCaP and PC-3 cells were less sensitive than DU145 cells to MSeA-induced apoptosis

We first validated the status of AKT and ERK1/2 in the three cell lines during exponential growth phase by western blot analysis (Figure 1A). As expected, LNCaP cells expressed androgen receptor (AR) and prostate specific antigen (PSA), whereas the two-androgen independent cell lines did not express either molecule. AKT (Ser473) phosphorylation was detected in the following order in the three cell lines: DU145 < LNCaP < PC-3. However, much higher levels of phospho-ERK1/2 were detected in DU145 cells than in LNCaP or PC-3 cells.

To compare their apoptotic sensitivity to MSeA, the cells were treated with increasing concentrations of MSeA for 24 h.

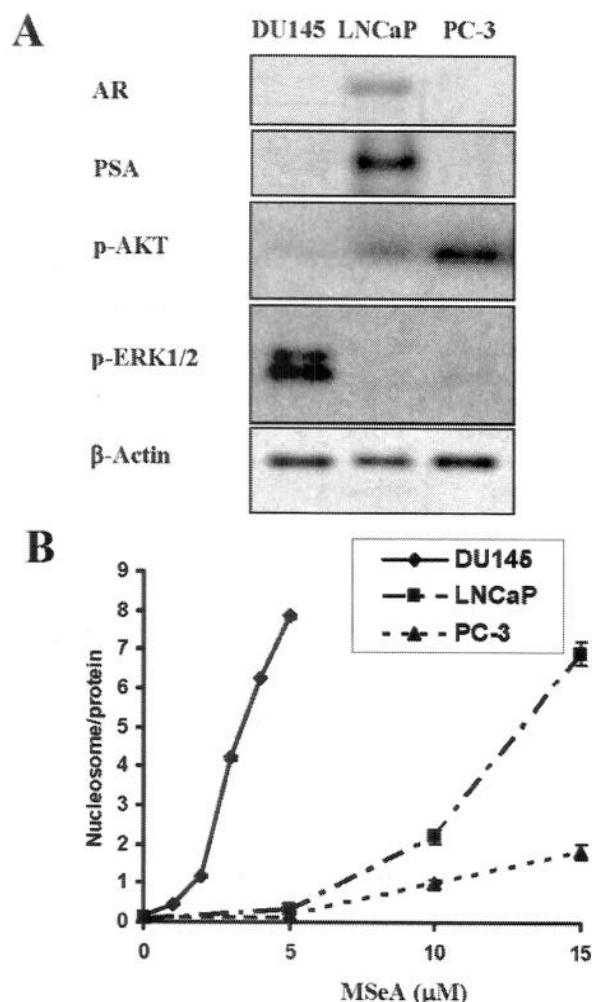


Fig. 1. (A) Immunoblot detection of AR, PSA and phospho-AKT and phospho-ERK1/2 in DU145, LNCaP and PC-3 cells lysate. (B) ELISA detection of apoptotic oligonucleosomes in DU145, LNCaP and PC-3 cells after 24 h treatment with increasing concentrations of MSeA. Floating and adherent cells were collected for apoptosis assay with cell death ELISA Kit. ELISA OD was normalized to protein content. Each point represents mean and SD of 4 replicates.

Apoptosis was estimated by Cell Death ELISA Kit. As shown in Figure 1B, a significant increase of apoptosis was observed in DU145 cells in response to MSeA exposure between 2 and 3 μ M. To elicit the same death response in LNCaP cells and PC-3 cells 4 and 7 times more MSeA were required, respectively. The apoptosis responses of the three cell lines showed an inverse association with their basal AKT phosphorylation levels.

LNCaP cells retained sensitivity to caspase-mediated apoptosis by selenite

To examine whether LNCaP cells are resistant to apoptosis induced by other forms of selenium, we compared the death response induced by MSeA with sodium selenite, which has been known to induce DNA damage and genotoxicity in several cell types (28–30). As shown in Figure 2A, while the caspase-mediated PARP cleavage was detected at a low level in LNCaP cells exposed to 10 μ M of MSeA (lane 5), 3 μ M of selenite was sufficient to induce significant PARP cleavage (lane 8). As a reference value, 5 μ M of selenite was needed to

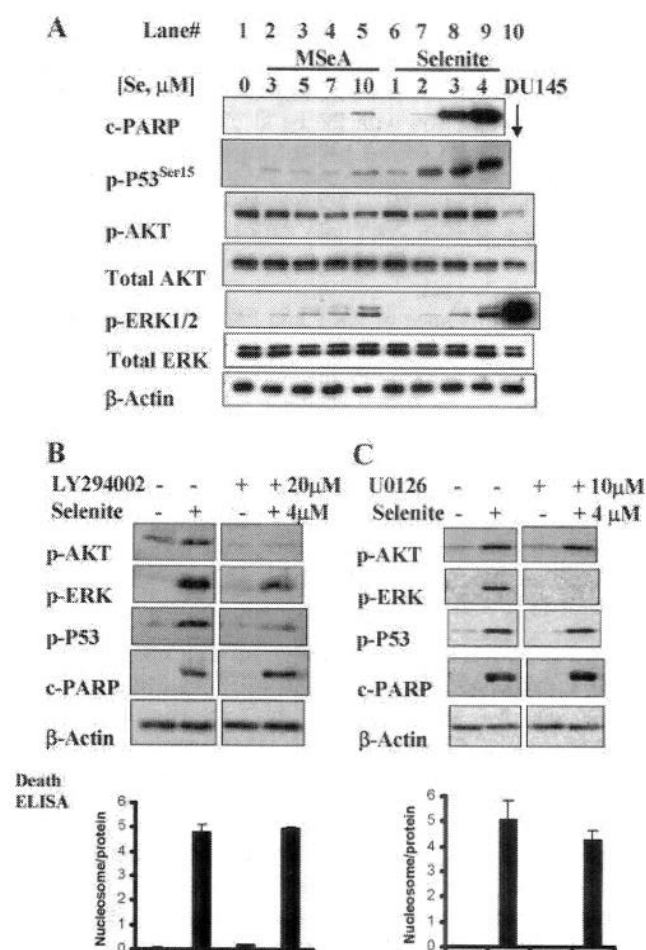


Fig. 2. (A) Immunoblot analyses of cleaved PARP (89 kDa) and the phosphorylation status of AKT and ERK1/2 in LNCaP cells after 24 h exposure to MSeA (lanes 2–5) and selenite (lanes 6–9). Lane 10 was DU145 cell lysate for comparison. β-Actin expression was probed to indicate the evenness of loading of the protein extract from each treatment. (B) Effect of PI3K inhibitor LY294002 and (C) effect of MEK inhibitor U0126 on caspase-mediated cleavage of PARP and overall death induced by selenite after 24 h exposure. Cells were pretreated with LY294002 or U0126 for 1 h before the addition of selenite for 24 h (total inhibitor exposure time was 25 h).

induce apoptotic DNA laddering in the absence of activation of caspases in DU145 cells (14). These results suggest that LNCaP cells possess mechanisms that suppress the MSeA-induced of caspase activation and apoptosis, and these mechanisms did not apply to the caspase activation pathways induced by selenite.

Effects of MSeA and selenite on phosphorylation status of AKT and ERK1/2

To probe the involvement of AKT and ERK1/2 pathways in regulating apoptosis induced by selenium in LNCaP cells, we assessed the effects of MSeA and selenite on the level of phospho-AKT and ERK1/2 after 24 h treatment (Figure 2A). MSeA exposure resulted in a dose-dependent yet modest decrease of AKT phosphorylation (lanes 2–5). Even with the highest dose of MSeA (lane 5), the remaining level of phospho-AKT was still much higher than in DU145 cells (lane 10). Contrary to the expectation based on our results in

DUI45 cells (14–16), MSeA treatment increased ERK1/2 phosphorylation in LNCaP cells in a dose-dependent manner (lanes 2–5), although the absolute magnitude of this increase was small in comparison with the basal phospho-ERK1/2 in DUI45 cells (lane 10). MSeA exerted minimal effect, if any, on p53 Ser15 phosphorylation (lanes 2–5). Selenite exposure at doses that led to significant PARP cleavage increased the phosphorylation of both AKT and ERK1/2 in LNCaP cells (lanes 8–9) and p53 Ser15 phosphorylation (lanes 7–9), as reported previously (34).

To test the significance of AKT and ERK phosphorylation during selenite-induced apoptosis, we examined the impact of PI3K inhibitor LY294002 (Figure 2B) and MEK inhibitor U0126 (Figure 2C) on PARP cleavage and overall death as detected by death ELISA. Whereas each inhibitor decreased the phosphorylation of the intended downstream target kinase, they did not significantly increase caspase-mediated apoptosis as indicated by the similar extent of cleavage of PARP or overall death. The results suggest that AKT and/or ERK1/2 do not play an important role in regulating apoptosis induced by selenite in LNCaP cells.

PI3K and MEK inhibitors sensitized LNCaP cells to MSeA-induced apoptosis

To test whether the sustained AKT activation and/or treatment-induced ERK activity in MSeA-exposed LNCaP cells decreased their sensitivity for apoptosis, we next examined the effects of PI3K inhibitor LY294002 and MEK inhibitor U0126, alone or in combination, on apoptosis induced by MSeA. As shown in Figure 3, LY294002 completely inhibited AKT phosphorylation (Figure 3B, lane 3 versus 1) and resulted in a small increase of apoptosis (Figure 3A, column 3 versus 1). Combining LY294002 with MSeA increased apoptosis ~4 times more than the sum of apoptotic signal induced by each agent alone (Figure 3A, column 4).

Treatment with U0126 abolished MSeA-induced ERK1/2 phosphorylation (Figure 3B, lane 6 versus 2). U0126 alone did not have any effect on background apoptosis (Figure 3A, column 5). Combining U0126 with MSeA increased apoptosis modestly (Figure 3A, column 6 versus 2) and the extent of death was about one-third of that induced by the LY294002/MSeA combination (column 6 versus 4).

When PI3K and MEK were inhibited simultaneously, cell death was comparable with that induced by PI3K inhibition alone (Figure 3A, column 7 versus 3). When the two inhibitors were combined with MSeA, we observed a super-enhancement of apoptosis (Figure 3A, column 8 versus 4 and 6). The enhanced apoptosis execution by either inhibitor or both combined did not involve an increase of p53 Ser15 phosphorylation (Figure 3B). Collectively, the data support sustained AKT activation in LNCaP cells as a key factor for suppressing apoptosis signaling from MSeA. Furthermore, MSeA-induced ERK1/2 activation provided an additional survival response to inhibit apoptosis signaling in LNCaP cells. Their effects did not involve p53 Ser15 phosphorylation.

Involvement of caspase-8 and caspase-9 cascades

To explore the caspase targets through which the PI3K and/or MEK inhibitor sensitized LNCaP cells to undergo MSeA-induced apoptosis, we examined the cleavage and activity of key caspases in both the intrinsic (caspase-9, Figure 3B) and extrinsic (caspase-8, Figure 3C) pathways (28,29). Caspase-3 cleavage patterns (Figure 3B) and activities (data not shown)

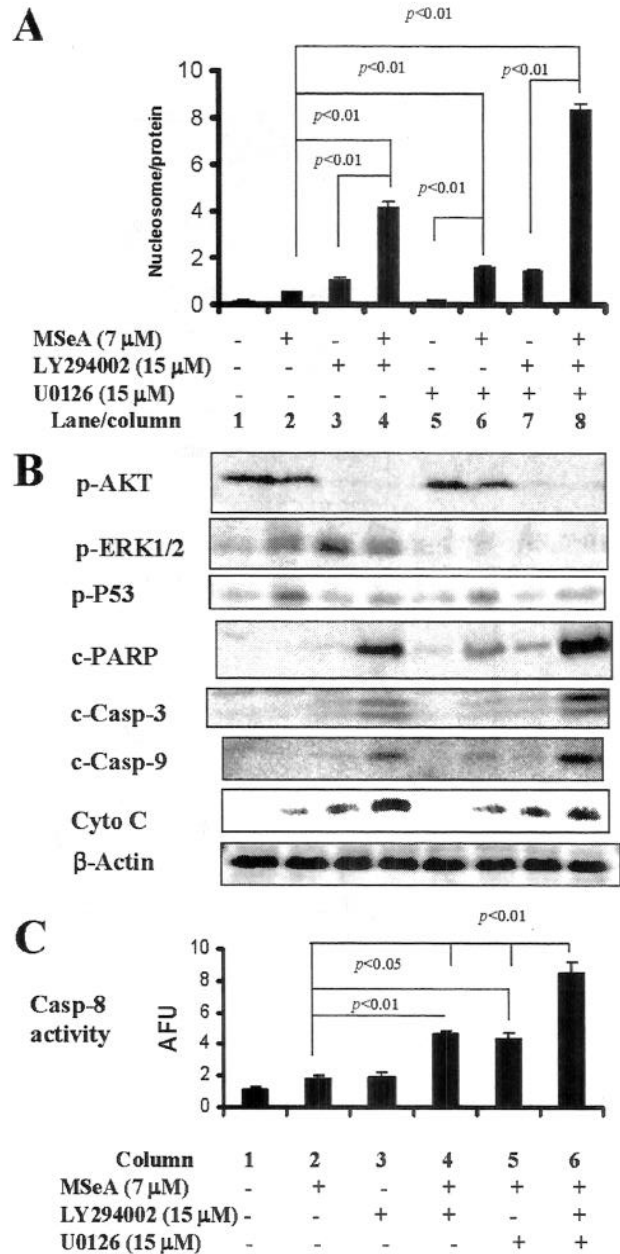


Fig. 3. (A) Effects of the PI3K inhibitor LY294002 and/or the MEK inhibitor U0126 on MSeA-induced apoptotic nucleosome release in LNCaP cells detected by ELISA. Cells were pretreated with LY294002 and/or U0126 for 1 h before the addition of MSeA for 24 h (total inhibitor exposure time was 25 h). Each column represents mean and SD of four replicates. (B) Immunoblot verification of inhibition of PI3K/AKT and MEK/ERK pathways by chemical inhibitors and detection of phospho-P53 Ser15, cleaved PARP, caspase-3 and caspase-9 and cytosolic cytochrome *c*. Experimental design was identical to (A). β -Actin expression was probed to indicate the evenness of loading of the protein extract from each treatment. (C) Caspase-8 activity assay in cells treated with MSeA and/or inhibitors. AFU, arbitrary fluorescence unit. Experimental design was identical to (A) except the omission of U0126 and U0126 plus LY294002 groups. Activity assay for caspase-3 and caspase-9 showed identical patterns as the cleavage patterns shown here in (B) (data not shown).

were in excellent agreement with the extent of PARP cleavage (Figure 3B) and with death ELISA data presented in Figure 3A, consistent with this executioner caspase as an ultimate mediator of PARP cleavage and DNA fragmentation in

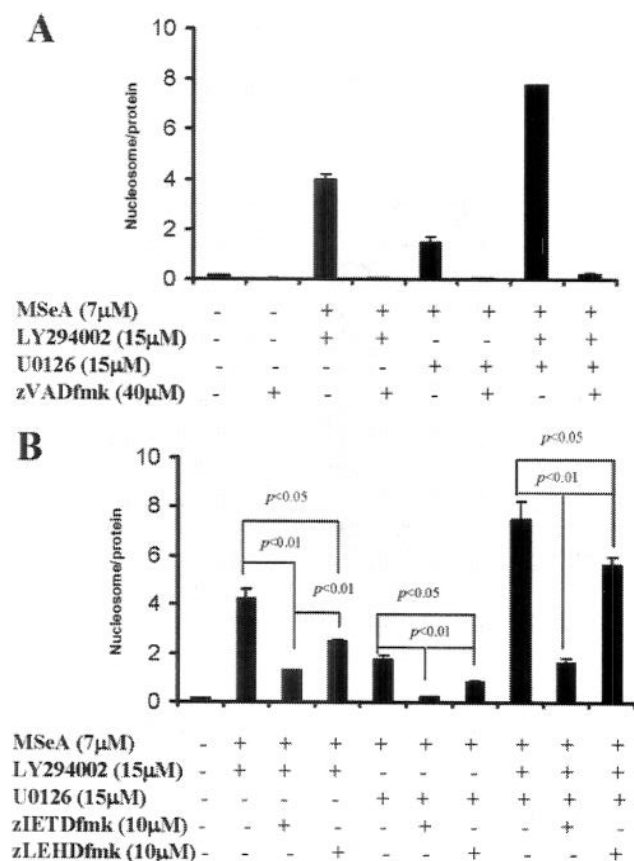


Fig. 4. Effects of (A) pan-caspase-inhibitor z-VAD-fmk and (B) caspase-8 inhibitor zIETDfmk or caspase-9 inhibitor zLEHDFmk on apoptosis induced by MSeA in combination with PI3K and/or ERK inhibitors in LNCaP cells. Exposure was for 24 h. Floating and adherent cells were combined for the death ELISA. Each column represents mean and SD of four replicates.

MSeA-induced apoptosis. The MSeA/LY294002 combination (Figure 3B, lane 4) and MSeA/U0126 combination (Figure 3B, lane 6) led to a similar enhancement of caspase-8 activity (Figure 3C, column 4 versus 5), yet their effects on caspase-9 and caspase-3 (Figure 3B, lane 4 versus 6) and apoptosis (Figure 3A, column 4 versus 6) differed by ~3 fold. This difference correlated to a lack of an effect of ERK inhibition on mitochondrial release of cytochrome *c* in MSeA-exposed cells (lane 6 versus 2). In contrast, PI3K/AKT inhibition led to a greater than additive increase in cytochrome *c* release in the MSeA-exposed cells (lane 4 versus 2). These results indicate that both PI3K/AKT and MEK/ERK suppressed caspase-8 activation, yet only the PI3K/AKT pathway exerted a major impact on cytochrome *c* release from mitochondria in MSeA-treated cells. This was further supported by the additive nature of caspase-8 activation when both pathways were inhibited (Figure 3C, column 6) without further enhancing cytochrome *c* release (Figure 3B, lane 8 versus 4).

To verify that the activation of caspases was crucial for apoptosis execution by MSeA in LNCaP cells when PI3K and/or MEK kinases were suppressed, we tested the effect of a general caspase inhibitor zVADfmk (40 µM) on apoptosis. As shown in Figure 4A, zVADfmk completely blocked the apoptosis induced by MSeA in combination with PI3K and/or MEK inhibitor. We next investigated the relative contribution of the two caspase cascades by using specific inhibitors.

As shown in Figure 4B, the caspase-8 inhibitor zIETDfmk blocked MSeA/LY-, MSeA/U0126- and MSeA/LY/U0126-induced death by ~70, ~95 and ~80%, respectively. However, the caspase-9 inhibitor zLEHDFmk at the same concentration of 10 µM only exerted ~40, ~50 and ~20% protective effects on the corresponding treatment combinations mentioned above. Taken together, the data indicate that the extrinsic caspase-8 cascade played a greater role than the intrinsic caspase-9 cascade in mediating MSeA-induced apoptosis in LNCaP cells when their PI3K/AKT and MEK/ERK1/2 activities were inhibited and that these two pathways regulated common (caspase-8) as well as distinct (mitochondria/cytochrome *c*) targets in the caspase activation cascades.

Effects of AKT inhibitors on MSeA-induced apoptosis in LNCaP cells

PI3K inhibition by LY294002 could be expected to and did decrease the activity of AKT as shown in Figures 2B and 3B, but could also affect the activities of other PI3K downstream target molecules. In order to establish a specific effect of AKT for regulating the apoptosis sensitivity of LNCaP to MSeA, we tested whether AKT inhibitors could similarly sensitize LNCaP cells to MSeA. NL-71-101, a modified PKA inhibitor that showed greater selective inhibition of AKT than its parental compound (35), exerted an enhancement action nearly identical to that of the PI3K inhibitor LY294002 (Figure 5A). The AKT-specific inhibitor, a 3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analogue (36), did not cause apoptosis at concentrations as high as 50 µM after 24 h exposure, but greatly sensitized LNCaP cells to MSeA-induced apoptosis in a dose-dependent manner (Figure 5B). Immunoblotting confirmed that this inhibitor significantly decreased the phospho-AKT level and increased caspase-3 cleavage activation and the cleavage of PARP (Figure 5C). These results indicated that AKT inhibition alone was insufficient to induce apoptosis in LNCaP cells within the duration of exposure of ~25 h, but was responsible for sensitizing LNCaP cells to MSeA.

Transfection of DU145 with an active AKT increased their resistance to MSeA

To test the prediction that upregulation of AKT activity in DU145 cells should make them more resistant to MSeA, we established stable transfectants expressing a CA-AKT and examined the effects on MSeA-induced apoptosis. Two clones (clones 8 and 11) expressing different levels of AKT activity (Figure 6A) were compared with vector-transfectants. As expected, the vector-transfectants remained sensitive to MSeA-induced apoptosis, whereas the cells transfected with CA-AKT became more resistant as indicated by fewer round-up cells (Figure 6B) and lower death ELISA readout in inverse proportion to their AKT levels (Figure 6C).

Discussion

Data presented above provided several lines of evidence supporting AKT as a key protein kinase for regulating the apoptosis sensitivity to MSeA in LNCaP and DU145 cells. The association of basal AKT activity with differential sensitivity of the three PCa cell lines (Figure 1) provided the initial clue. The sensitization of LNCaP cells to undergo MSeA-induced apoptosis through attenuating AKT activity with the PI3K inhibitor LY294002 (Figures 3 and 4), with a PKA

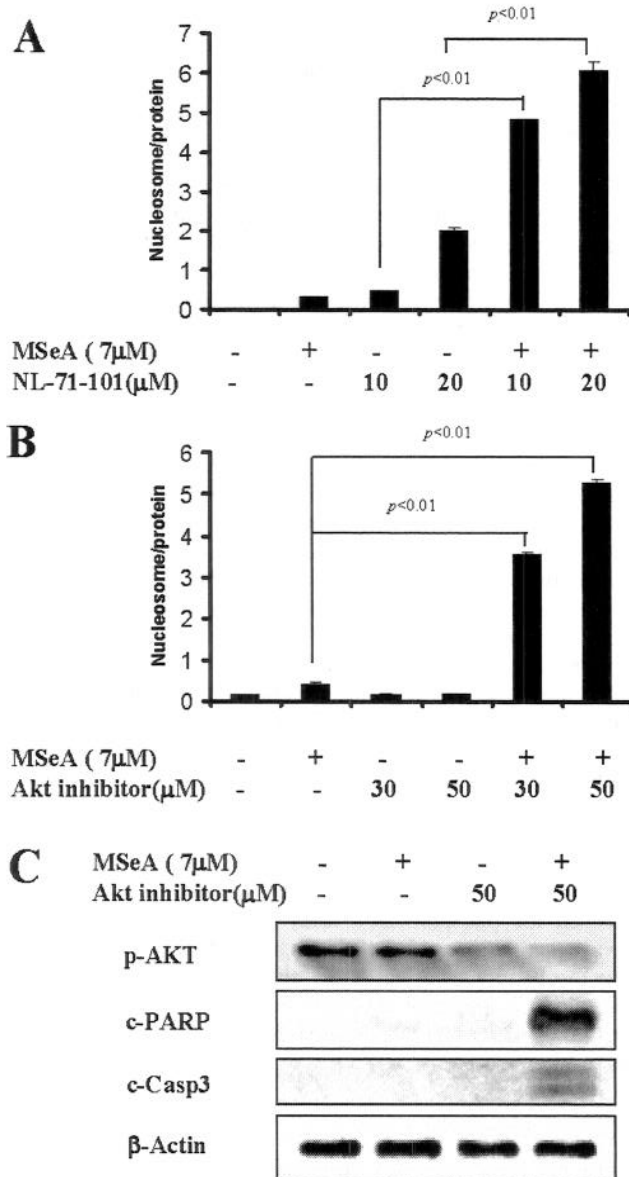


Fig. 5. Effects of AKT inhibitors on apoptosis induced by MSeA in LNCaP cells. (A) PKA inhibitor-based AKT inhibitor NL-71-101 (Catalog No. 487940, CalBiochem, La Jolla, CA). (B) and (C) Akt specific inhibitor [3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analog, Catalog No. 124005, CalBiochem, La Jolla, CA]. Cells were pretreated with the inhibitors for 1 h before the addition of MSeA for 24 h (total inhibitor exposure time was 25 h). Floating and adherent cells were combined for the death ELISA (B) and for immunoblotting analyses (C). Each column represents mean and SD of four replicates.

inhibitor-derived AKT inhibitor NL-71-101 (35) (Figure 5A) and with its specific 3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analog inhibitor (36) (Figure 5B) together made a compelling case. Furthermore, overexpression of a CA-AKT rendered DU145 cells more resistant to MSeA in direct proportion to the AKT activity of the transfectant clones (Figure 6). Therefore, irrespective of the androgen dependence status or p53 function (wild type in LNCaP, mutant in DU145), AKT activity can regulate apoptosis induced by MSeA in these cells. In further support of the assertion on p53 independence for MSeA-induced death, p53

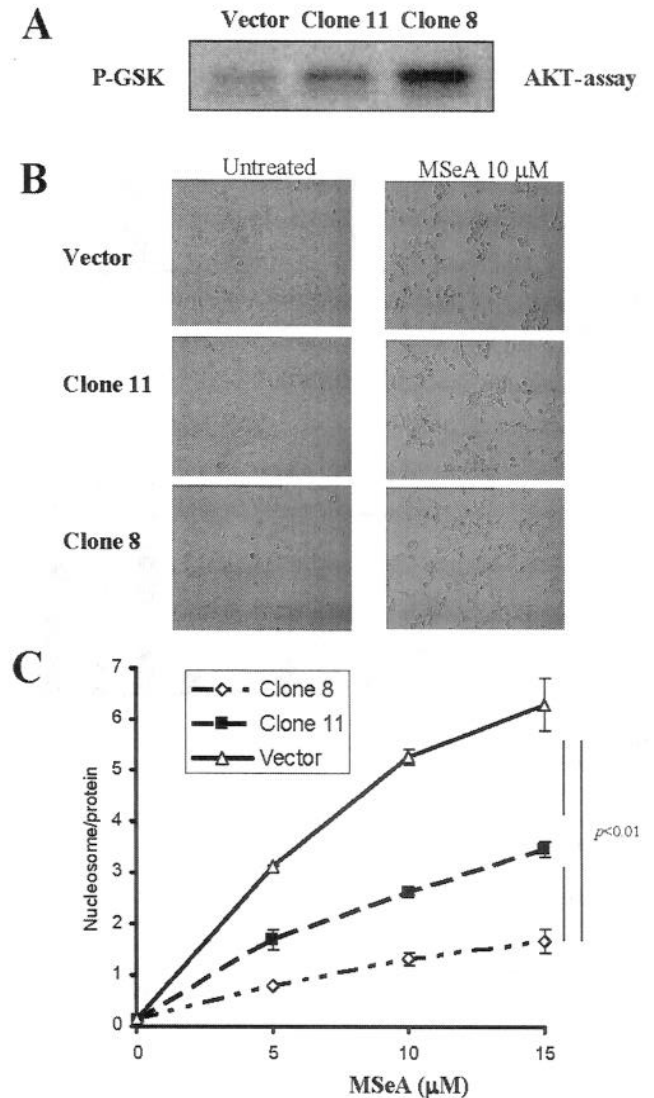


Fig. 6. Effect of expression of a CA-AKT in DU145 cells on MSeA-induced apoptosis. (A) AKT activity assay for cell lysate of vector- and AKT-transfectants. Phosphorylation of GSK-fusion protein substrate by immunoprecipitated AKT was detected by immunoblot. (B) Phase contrast photomicrograph of morphological responses of the different clones to MSeA after 16 h exposure. 100× magnification. (C) Induction of apoptosis by MSeA in the transfectant clones detected by death ELISA (24 h exposure). Each point represents mean and SD of four replicates. These data are representative of at least two independent experiments.

Ser15 phosphorylation was not changed during the enhanced apoptosis execution by inhibitors of either AKT or ERK pathway (Figure 3B).

A second mitigating factor is the unexpected induction of ERK1/2 activation by MSeA, possibly an effect unique to the LNCaP cells because MSeA exposure decreases ERK1/2 phosphorylation in DU145 cells (14–16). Blocking ERK1/2 activation with U0126 slightly enhanced MSeA induction of apoptosis and resulted in a super-synergy when combined with the PI3K inhibitor (Figure 3). ERK1/2 activation therefore appears to be a compensatory survival response that offsets the apoptotic signaling induced by MSeA in LNCaP cells. The reasons for the two cell lines to show opposite patterns of ERK1/2 response to MSeA are not clear and require further

investigation. In contrast to the significant regulatory effects of AKT and ERK on MSeA-induced apoptosis in LNCaP cells, these kinases exerted little influence on apoptosis induced by selenite (Figure 2). These findings support the involvement of different protein kinase pathways for regulating apoptosis induction by different forms of selenium in PCa and other cell types (16). Such differences were also reflected in the caspase cascades activated as discussed next.

Our data showed that the general caspase inhibitor zVADfmk completely blocked apoptosis induced by MSeA in combination with PI3K and/or ERK inhibitor (Figure 4A), suggesting that AKT and ERK1/2 conferred resistance to MSeA-induced apoptosis ultimately through suppressing caspase activation pathways in LNCaP cells. In this scenario, when the suppression was relieved by one or both kinase inhibitors, MSeA-initiated signaling to caspase-8, caspase-9 and caspase-3 went through in variable degrees depending on which barrier was removed. The caspase cleavage patterns and activity assay results (Figure 3B and C) support a greater inhibition by AKT of mitochondria/cytochrome *c*/caspase-9 cascade than by ERK1/2 even though both kinase pathways attenuated caspase-8 activity with similar potency. The results of specific caspase inhibitors (Figure 4B) demonstrated that blocking caspase-8 pathway exerted a much greater protective effect against apoptosis induction than blocking caspase-9 pathway. This pattern of caspase activation by MSeA in LNCaP cells is essentially identical to that induced by MSeA in DU145 cells, wherein caspase-8 is a major initiator caspase upstream of caspase-9 (14). In contrast to these patterns, selenite-induced caspase-mediated apoptosis in LNCaP cells equally involved these two caspase cascades and was causally linked to p53 Ser15 phosphorylation, as we reported previously (34).

Our data suggest that AKT and ERK1/2 probably target different molecules in the extrinsic (major) and intrinsic (likely subordinate to caspase-8) caspase activation cascades to inhibit apoptosis induced by MSeA in LNCaP cells. Potential targets of AKT- and/or ERK1/2-mediated suppression of caspase pathways include an upregulation of the caspase-8 inhibitory proteins FLIPs by AKT (37,38) and ERK (39), AKT-mediated phosphorylation of BAD at serine136 (40,41), ERK-mediated phosphorylation of BAD at serine112 (42), AKT-mediated phosphorylation inactivation of caspase-9 (43), AKT-mediated phosphorylation inhibition of a Forkhead transcription factor (44) and AKT-induced expression of caspase-3 inhibitor protein survivin (45–47), to name a few. Some of these targets directly affect the activity of multiple caspases, and others impact mitochondria integrity and the intrinsic cascades. AKT has a number of targets affecting the intrinsic pathway and therefore was not surprising that we observed greatly enhanced mitochondrial release of cytochrome *c* by PI3K/AKT inhibition while MEK/ERK inhibition had no effect (Figure 3B). The actual target molecules for AKT and ERK pathways to regulate MSeA-induced apoptosis in PCa cells are being investigated.

In summary, the AKT activity played a critical role in regulating the sensitivity of LNCaP and DU145 PCa cells to the induction of apoptosis by MSeA. The MSeA-induced activation of ERK1/2 constituted an additional and possibly unique survival response that further rendered LNCaP cells less sensitive to MSeA. These kinase pathways conferred resistance by ultimately suppressing caspases, including both caspase-8 (major) and caspase-9 (minor) cascades, independent of p53.

If applicable *in vivo*, our findings suggest that PCa cells with deregulated PTEN may be less susceptible to apoptosis by methylselenol, and combination with other agents that inhibit the PI3K-AKT signaling may improve overall chemoprevention efficacy. It is also possible that in a therapeutic context, targeted therapy through a combined use of methylselenium and PI3K/AKT inhibitors may improve the therapeutic outcome based on a prior knowledge of the PTEN-AKT profile of the tumors to be treated.

Acknowledgements

The authors thank Dr Zigang Dong for generously providing the AKT plasmid and Dr Howard Ganther for supplying MSeA. Supported in parts by Hormel Foundation, Eagles' Telethon, by grants from the National Cancer Institute (CA92231 and CA95642) and Department of Defense Prostate Cancer Research Program (DAMD17-02-1-0007).

Conflict of Interest Statement: None declared.

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Received December 21, 2004; revised March 30, 2005;
accepted April 10, 2005

Methylseleninic Acid Potentiates Apoptosis Induced by Chemotherapeutic Drugs in Androgen-Independent Prostate Cancer Cells

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ABSTRACT

Purpose: To test whether and how selenium enhances the apoptosis potency of selected chemotherapeutic drugs in prostate cancer (PCA) cells.

Experimental Design: DU145 and PC3 human androgen-independent PCA cells were exposed to minimal apoptotic doses of selenium and/or the topoisomerase I inhibitor 7-ethyl-10-hydroxycamptothecin (SN38), the topoisomerase II inhibitor etoposide or the microtubule inhibitor paclitaxel/taxol. Apoptosis was measured by ELISA for histone-associated DNA fragments, by flow cytometric analysis of sub-G₁ fraction, and by immunoblot analysis of cleaved poly(ADP-ribose)polymerase. Pharmacologic inhibitors were used to manipulate caspases and c-Jun-NH₂-terminal kinases (JNK).

Results: The methylselenol precursor methylseleninic acid (MSeA) increased the apoptosis potency of SN38, etoposide, or paclitaxel by several folds higher than the expected sum of the apoptosis induced by MSeA and each drug alone. The combination treatment did not further enhance JNK1/2 phosphorylation that was induced by each drug in DU145 cells. The JNK inhibitor SP600125 substantially decreased the activation of caspases and apoptosis induced by MSeA combination with SN38 or etoposide and completely blocked these events induced by MSeA/paclitaxel. The caspase-8 inhibitor zIETDfmk completely abolished apoptosis and caspase-9 and caspase-3 cleavage, whereas the caspase-9 inhibitor zLEHDfmk significantly decreased caspase-3 cleavage and apoptosis but had no effect on caspase-8 cleavage. None of these caspase inhibitors abolished JNK1/2 phosphorylation. A JNK-independent suppression of survivin by SN38 and etoposide, but not by paclitaxel, was also observed. In contrast to MSeA, selenite

did not show any enhancing effect on the apoptosis induced by these drugs.

Conclusions: MSeA enhanced apoptosis induced by cancer therapeutic drugs in androgen-independent PCA cells. In DU145 cells, the enhancing effect was primarily through interactions between MSeA and JNK-dependent targets to amplify the caspase-8-initiated activation cascades. The results suggest a novel use of methyl selenium for improving the chemotherapy of PCA.

INTRODUCTION

In North America, prostate cancer (PCA) is the most prevalent cancer diagnosed each year and the second leading cause of cancer-related death in older men (1). The death toll from PCA is quite heavy, accounting for an estimated loss of 30,000 Americans in 2004 (1). For PCA confined within the prostate, surgery and/or radiation therapy are curative in most cases (2, 3). Systemic chemotherapy is often the only palliative treatment option for managing advanced metastatic PCA that has failed surgery, radiation, and hormonal therapies. Due to a low proliferation rate, PCA is not highly responsive to standard chemotherapeutics that usually target proliferating cells. Furthermore, the drugs in use today are relatively nonselective and often manifest dose-limiting toxicity in normal tissues. A major challenge to improving their chemotherapeutic index is by selectively increasing the cancer cell-killing action of the drug and at the same time, reducing its systemic toxicity. To this end, Cao et al. (4) recently showed that pretreatment with selenium not only increases the cure rate of the topoisomerase I poison CPT-11, also known as irinotecan, in mice bearing human colon or head and neck cancer xenograft, but also decreases the lethality of this and other anticancer drugs.

Preclinical studies have shown that high levels of selenium can induce caspase-mediated and caspase-independent apoptosis of PCA cells (5–8). This mechanism may sensitize cancer cells to chemotherapeutic drugs. In the present study, we investigated whether selenium could enhance the apoptotic efficacy of several cancer therapeutic drugs in human prostate carcinoma DU145 and PC-3 cell lines. These cell lines are androgen-independent and represent advanced metastatic PCA against which current chemotherapeutic regimens have limited efficacy. We have previously used the DU145 cell model to define the caspase-mediated apoptotic response induced by different selenium compounds (5–7). Methylseleninic acid (MSeA) was used in the present study to investigate selenium/drug interaction because it was developed specifically for *in vitro* experiments as a penultimate precursor to the active selenium metabolite, methylselenol (9, 10). We chose 7-ethyl-10-hydroxycamptothecin (SN38), the active metabolite of CPT-11, etoposide/VP-16 and paclitaxel/taxol as representatives of two classes of anticancer drugs with entirely different mechanisms of action. SN38 and etoposide are inhibitors of topoisomerases

Received 10/12/04; revised 12/1/04; accepted 12/15/04.

Grant support: Supported by the Hormel Foundation and by grants CA92231 and CA95642 from the National Cancer Institute (J. Lü) and Department of Defense Prostate Cancer Research Program grant DAMD17-02-1-0007 (J. Lü).

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I and II, respectively. The advantage of using SN38 instead of the prodrug CPT-11 in cell culture is that the latter requires activation by carboxyesterase to generate SN38 (11). Topoisomerase poisons cause DNA double-strand breaks due to the inhibition of cleavable DNA/topoisomerase complexes during DNA replication (12), thereby leading to S phase arrest and apoptosis. Paclitaxel is an antimicrotubule drug and is clinically used for the treatment of prostate, breast, and other cancers (13, 14). Paclitaxel is known to induce G₂-M arrest, mitotic catastrophe and phosphorylative inactivation of Bcl-2, which in turn might stimulate mitochondria-driven apoptosis (14, 15). All three drugs have been shown to induce the stress activated c-Jun-NH₂-terminal kinase (JNK) pathway in many cancer cells including DU145 cells and induce caspase-mediated apoptosis which is often JNK-dependent (16–23). There is no published literature documenting the involvement of JNK pathway in apoptosis induced by MSeA. The major objectives of the study were to examine whether MSeA could magnify the activation of the initiator and executioner caspases in the presence of a chemotherapeutic drug, and whether the JNK pathway plays a key role in this process.

MATERIALS AND METHODS

Chemicals and Reagents. MSeA (CH₃SeO₂H) was synthesized as previously described (9, 10). Sodium selenite pentahydrate was purchased from J.T. Baker, Inc., Phillipsburg, NJ. SN38 was obtained from Pharmacia Upjohn/Pfizer (Kalamazoo, MI). Paclitaxel and etoposide and an antibody for β -actin were purchased from Sigma Chemical Co., St. Louis, MO. The general caspase inhibitor (zVADfmk), the specific inhibitors for caspase-8 (zIETDfmk), caspase-9 (zLEHDfmk), and caspase-3/-7 (zDEVDfmk) were purchased from MP-Biomedicals, Inc., Aurora, OH. The protein kinase inhibitors SB202190 (for p38MAPK) and SP600125 (for JNK1/2) were purchased from Calbiochem, La Jolla, CA. Antibodies specific for survivin, XIAP, cleaved poly(ADP-ribose)polymerase (PARP; p89), cleaved caspases-3, -8 and -9, as well as antibodies for total and phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) and p38MAPK (Thr¹⁸⁰/Tyr¹⁸²) were purchased from Cell Signaling Technology, Beverly, MA.

Cell Culture and Treatments. DU145 and PC3 cell lines were obtained from the American Type Culture Collection, Manassas, VA. DU145 cells were grown in MEM supplemented with 10% fetal bovine serum without antibiotics. PC-3 cells were grown in F-12K medium with 10% fetal bovine serum without antibiotics. At 48 hours after plating when cells were 50% to 60% confluent, the medium was changed before starting the treatment with MSeA or the other agents. To standardize all MSeA/drug exposure conditions, cells were bathed in culture medium at a volume to surface area ratio of 0.2 mL per cm² (e.g., 15 mL for a T75 flask and 5 mL for a T25 flask). For the experiments in which JNK and p38MAPK inhibitors were used, the cells were exposed to the inhibitors 1 hour prior to initiating treatment with SN38 and MSeA. For the experiments in which caspase inhibitors were used, the inhibitors and the drugs were given to the cells at the same time. DMSO (2 μ L/mL or less) was added as a vehicle solvent to the control culture that did not receive the inhibitor. This concentration of DMSO did not cause any adverse morphologic response.

Apoptosis Evaluation. Apoptosis was assessed by three methods. The first was a cell death detection ELISA kit purchased from Roche Diagnostics Corporation, Indianapolis, IN. This assay detects oligonucleosomes released after gentle lysis of the cell. Cells were cultured in T25 flasks for the desired duration. The spent medium containing floating cells was saved and kept on ice. The adherent cells were collected by gentle trypsinization and were combined with the floaters for pelleting by centrifugation. After gentle lysis of the cells with the buffer provided with the detection kit, the cell lysate was used for the ELISA test. The results were normalized by the protein content which was determined by the Lowry method using a reagent kit from Sigma. The second method was immunoblot analysis of PARP cleavage as described previously (5). The third assay was by flow cytometric analysis of apoptotic sub-G₁ fraction of 70% ethanol fixed cells with propidium iodide staining using a Becton Dickinson flow cytometer. Cells/apoptotic bodies with DNA content below 10% of G₁ DNA content were excluded from the calculated death rate.

Immunoblot Analyses. Both floating and attached cells were harvested as described above. The cell pellet was washed in PBS twice and the lysate was prepared in radioimmunoprecipitation assay buffer as described previously (5). Immunoblot analyses were essentially as described (5), except that the signals were detected by enhanced chemofluorescence with a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Methylseleninic Acid/Drug Combination Markedly Enhanced Apoptosis Compared to the Single Agent. In order to increase the likelihood of detecting a magnified response to the combination, we decided to use doses of MSeA or the drugs that by themselves would only minimally induce apoptosis in DU145 cells. Based on our preliminary dose-range finding experiments, we chose dose levels of 1 to 2 μ mol/L MSeA, 10 to 20 nmol/L SN38 or paclitaxel, and 15 to 20 μ mol/L etoposide for the combination treatment. As shown in Fig. 1A, exposure to 1 μ mol/L MSeA for 24 hours did not increase apoptotic DNA fragmentation detected by the Death ELISA kit (column 2 versus 1). In contrast, SN38 at 10 or 20 nmol/L induced modest but dose-dependent, increases of apoptosis (columns 3 and 4). Combining MSeA with SN38 increased apoptotic DNA fragmentation by ~4-fold over the sum achieved by the single agent treatment (columns 5 and 6 versus columns 3 and 4).

To confirm the enhanced apoptosis induced by SN38/MSeA combination, we carried out flow cytometric analysis of the apoptotic sub-G₁ fraction in the treated cells after 24 hours of exposure (Fig. 1B). Based on three independent experiments, the sub-G₁ fraction was as follows (mean $\pm$ SD; $n = 3$): untreated cells, 1.2 $\pm$ 0.4%; cells treated with 2 μ mol/L MSeA, 2.6 $\pm$ 0.7%; cells treated with 10 nmol/L SN38, 5.7 $\pm$ 2.1%; cells treated with the 2 μ mol/L MSeA and 10 nmol/L SN38 combination, 16.9 $\pm$ 1.8%. In terms of cell cycle effects, exposure of DU145 cells to the low level of MSeA did not exert a significant effect on cell distribution in the different phases. On the other hand, SN38 treatment led to a significant early S phase arrest consistent with topoisomerase I poisoning of cells

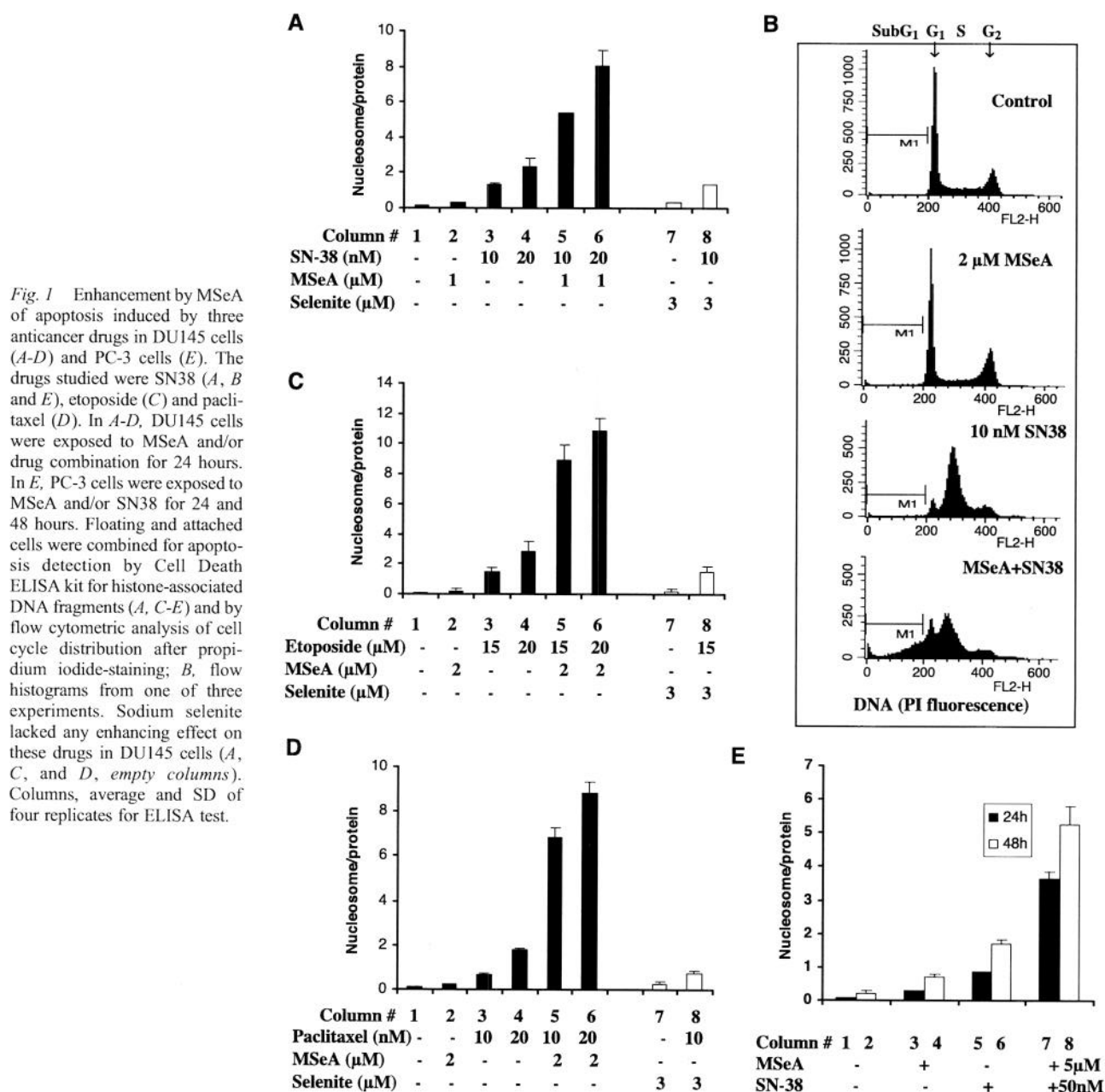


Fig. 1 Enhancement by MSeA of apoptosis induced by three anticancer drugs in DU145 cells (*A-D*) and PC-3 cells (*E*). The drugs studied were SN38 (*A, B* and *E*), etoposide (*C*) and paclitaxel (*D*). In *A-D*, DU145 cells were exposed to MSeA and/or drug combination for 24 hours. In *E*, PC-3 cells were exposed to MSeA and/or SN38 for 24 and 48 hours. Floating and attached cells were combined for apoptosis detection by Cell Death ELISA kit for histone-associated DNA fragments (*A, C-E*) and by flow cytometric analysis of cell cycle distribution after propidium iodide-staining; *B*, flow histograms from one of three experiments. Sodium selenite lacked any enhancing effect on these drugs in DU145 cells (*A, C*, and *D*, empty columns). Columns, average and SD of four replicates for ELISA test.

engaged in DNA replication. Combining MSeA with SN38 decreased the proportion of cells stuck in early S phase and increased the sub-G₁ fraction, suggesting that enhanced apoptosis occurred primarily in cells stuck in S phase by SN38.

The same pattern of augmented apoptosis was observed with the MSeA and etoposide combination (Fig. 1C). In brief, etoposide at 15 or 20 μmol/L induced small increases in apoptosis, but this effect was greatly magnified in combination with 2 μmol/L MSeA. Similar to SN38, flow cytometric analyses showed an early S phase arrest by etoposide (data not shown). Because SN38 and etoposide target topoisomerase I and II, respectively, and cause DNA strand breaks, we extended our study to the microtubule drug paclitaxel in order to determine the universality of this phenomenon. As shown in Fig. 1D, the

combination of MSeA and paclitaxel also produced a much greater effect on apoptosis compared with paclitaxel alone. Cell cycle analyses showed a strong G₂-M arrest by paclitaxel, as expected from its microtubule targeting action, and MSeA did not affect this cell cycle arrest action of paclitaxel (data not shown).

Furthermore, the enhancement action of MSeA on drug-induced apoptosis was not unique to DU145 cells because a similar augmentation of apoptosis was detected in PC-3 cells for SN38 (Fig. 1E) and the two other drugs (data not shown). The consistency of the data with the three drugs points to some common mechanism underlying the enhancing effect of MSeA and this effect was independent of the cell cycle arrest actions (regardless of S arrest or G₂-M arrest) of the drugs.

Selenite did not Enhance Apoptosis Induced by 7-Ethyl-10-Hydroxycamptothecin, Etoposide, or Paclitaxel. The chemical specificity of selenium in potentiating the effect of these drugs was evaluated by using sodium selenite in DU145 cells. As shown on the right hand portion of Fig. 1*A*, *C*, and *D*, selenite at 3 $\mu\text{mol/L}$ did not induce apoptosis (*column 7*), nor did it potentiate the apoptotic response to 10 nmol/L SN38, 15 $\mu\text{mol/L}$ etoposide, or 10 nmol/L paclitaxel (compare *column 8* versus *column 3* in *A*, *C* and *D*). These results support the unique attribute of a monomethylated selenium metabolite as exemplified by MSeA for enhancing drug-induced apoptosis in PCA cells.

Effect of Treatment Scheduling on the Interaction Between Methylseleninic Acid and 7-Ethyl-10-Hydroxycamptothecin. Next, we used SN38 as a prototype drug to examine how different treatment schedules with MSeA might impact on the apoptosis potency of SN38 in DU145 cells. In the following experiments, the treated cells were harvested for

evaluation with the death ELISA kit after SN38 exposure for 24 hours. As shown in Fig. 2*A*, the simultaneous treatment with SN38 and MSeA for 24 hours (*regimen b*) was the protocol in the previous experiments. Pretreatment with MSeA first for 24 hours followed by simultaneous treatment with SN38 and MSeA for 24 hours (*regimen a*) resulted in a slightly diminished response compared with that of *regimen b*. The sequential protocol of pretreatment with MSeA for 24 hours followed by SN38 only for an additional 24 hours (*regimen c*) failed to show any potentiation effect. However, when MSeA was introduced after SN38 treatment had been initiated for 12 hours (*regimen d*), a significant enhancement was still observed, although it was not as dramatic as that produced by the simultaneous treatment with both agents (*regimen b*). These results suggest that the maintenance of MSeA at some critical level is necessary in order to maximize the apoptotic response to SN38. In this regard, it is important to point out that MSeA can be depleted within 24 hours in culture medium (24).

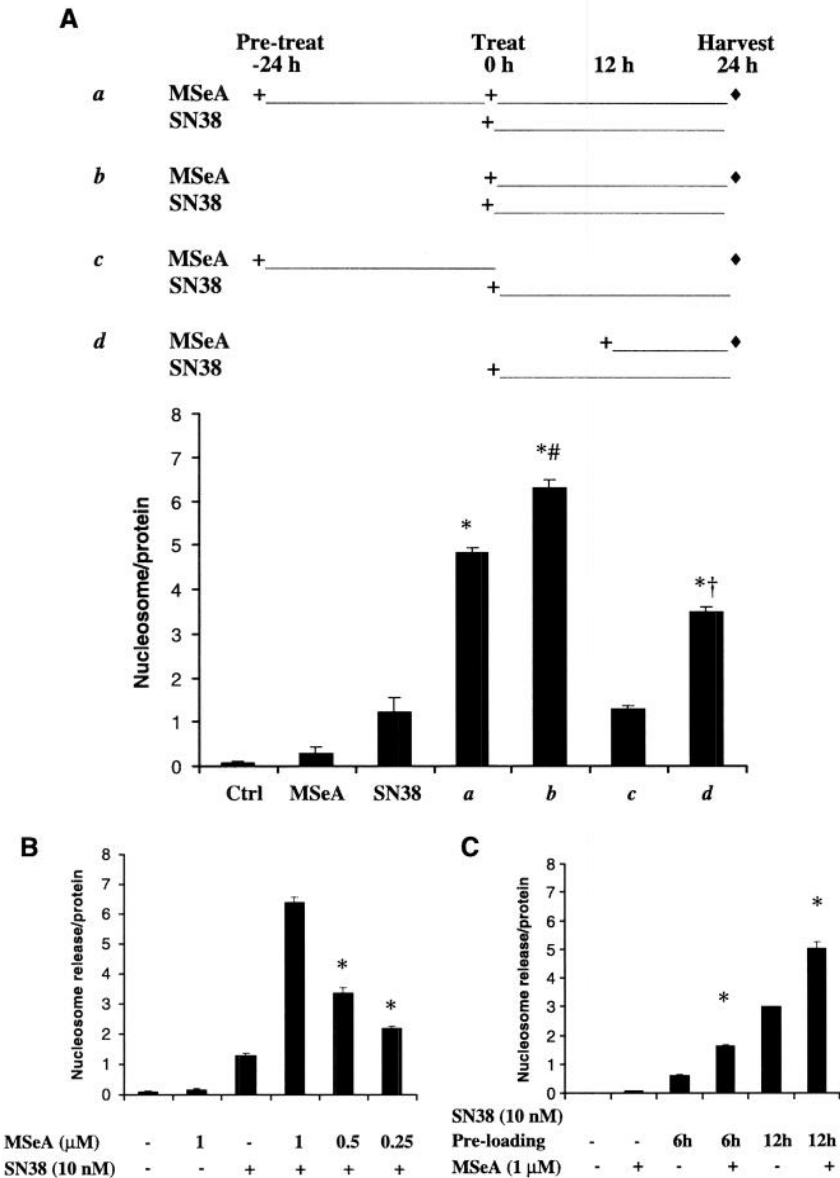


Fig. 2 *A*, effects of the dosing sequence (regimen) between SN38 and MSeA on the extent of apoptosis in DU145 cells. Dosing regimens (*a-d*) were as sketched above the plot. Treatment concentration used was 1 $\mu\text{mol/L}$ MSeA and 10 nmol/L SN38, respectively. *, $P < 0.05$ compared with the SN38 treatment alone; #, $P < 0.05$ compared with regimen *a* treatment; †, $P < 0.05$ compared with regimen *b* treatment ($n = 4$ replicates); *B*, determination of the MSeA threshold concentration that enhanced the apoptosis potency of SN38. DU145 cells were cotreated with 10 nmol/L SN38 and the indicated concentrations of MSeA for 24 hours; *C*, effect of MSeA on apoptosis signaling initiated by preloaded SN38. DU145 cells were treated with 10 nmol/L SN38 for 6 hours or 12 hours. The medium was removed and the cells were washed with serum-free medium to remove residual SN38. Fresh medium with or without 1 $\mu\text{mol/L}$ MSeA were added for 24 hours and apoptosis was assessed by ELISA; *, $P < 0.05$ compared with the SN38 treatment alone ($n = 4$ replicates).

To test the notion of a threshold requirement for MSeA, we varied the concentration of MSeA although holding SN38 constant at 10 nmol/L and carried out the experiment using the simultaneous treatment protocol of *regimen b*. As shown in Fig. 2B, MSeA dose-dependently enhanced the apoptosis response to SN38. As little as 0.5 $\mu\text{mol/L}$ MSeA was able to magnify the apoptotic effect of SN38 in DU145 cells, even though the augmentation was decidedly subdued with the lower doses.

A possibility for the simultaneous treatment with MSeA and SN38 to produce greatest enhancement on apoptosis is that MSeA may increase the cellular uptake/retention of SN38. Therefore, we did a wash-out experiment to study the effect of MSeA on apoptosis initiated by preloaded SN38 without this potential complication. Flasks of DU145 cells were treated with SN38 for 6 or 12 hours. The medium was removed and the cells were washed with serum-free medium to remove the residual SN38. The cells were given fresh media with or without 1 $\mu\text{mol/L}$ MSeA for an additional 24 hours and harvested for apoptosis ELISA test. As shown in Fig. 2C, addition of MSeA to cells preloaded with SN38 still enhanced apoptosis by ~ 2 -fold. These data indicate that MSeA can amplify SN38-initiated death signaling independent of an effect, if any, on SN38 uptake/retention.

Inhibition of c-Jun-NH₂-Kinase, but not p38MAPK, Greatly Attenuated 7-Ethyl-10-Hydroxycamptothecin/Methylseleninic Acid-Induced Apoptosis. In order to obtain insight into the signaling pathway(s) that might play a role in the potentiation action of MSeA on SN38 potency, we focused on the stress-activated protein kinases, JNK1/2 and p38MAPK. DU145 cells were treated with SN38 and/or MSeA for 8 or 24 hours with the simultaneous exposure protocol. Immunoblot analysis of the phosphorylation (i.e., activation) status of these kinases or of the JNK substrate c-Jun showed that JNK1/2 and p38MAPK phosphorylation levels were increased significantly by SN38 alone at 24 hours (Fig. 3A, lane 7 versus 5), but not at 8 hours (Fig. 3A, lane 3 versus 1). These effects were observed without any change in the expression of total JNK1/2 or total p38MAPK. In a subsequent time course experiment, SN38-induced phosphorylation of JNK1/2 was also detected at 12 and 16 hours of treatment (data not shown). Thus, there was apparently a delayed but sustained activation of JNK1/2 beginning between 8 and 12 hours of SN38 exposure. MSeA alone had no effect on phospho-JNK1/2 or phospho-p38MAPK (lanes 2 and 6). The combination of MSeA and SN38 did not increase the levels of phospho-JNK1/2, phospho-c-Jun, or phospho-p38MAPK when compared with SN38 alone (Fig. 3A, lane 8 versus 7).

To determine the functional significance of the phosphorylation of JNK1/2 and p38MAPK in SN38/MSeA-induced apoptosis, we used an inhibitor of JNK1/2, SP600125, and an inhibitor of p38MAPK, SB202190, to block their activation. As shown in Fig. 3A, SP600125 effectively decreased SN38/MSeA-induced JNK1/2- and c-Jun phosphorylation, but had no effect on p38MAPK phosphorylation (lane 9 versus 8), thus confirming the selectivity of the inhibitor. Additionally, the JNK inhibitor resulted in a marked decrease ($\sim 70\%$) of SN38/MSeA-induced apoptosis (Fig. 3B, column 5 versus 4). On the other hand, inhibiting

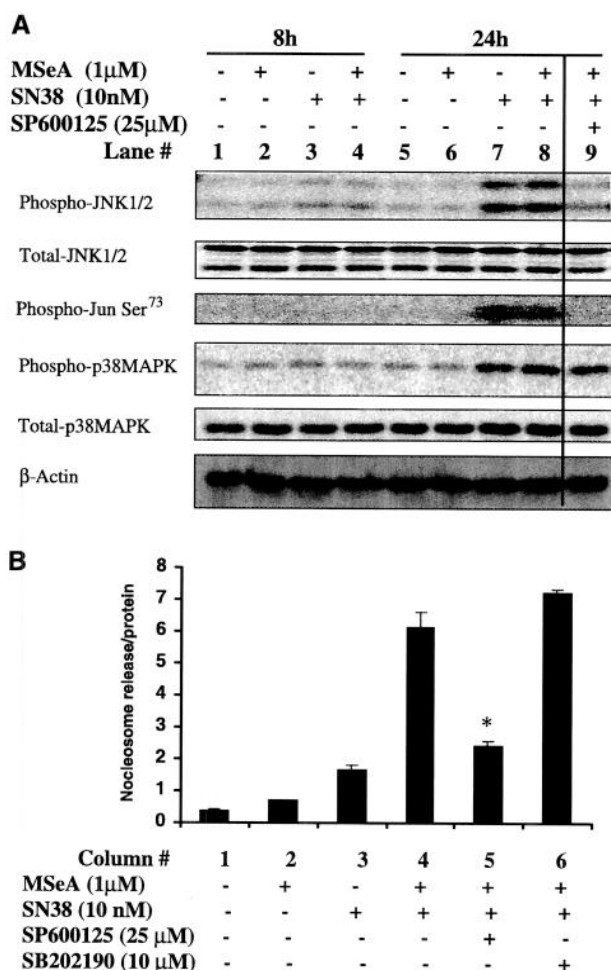


Fig. 3 A, immunoblot detection of phosphorylated JNK1/2, p38MAPK, and c-Jun in DU145 cells at 8 or 24 hours of exposure to SN38 and/or MSeA and the verification of JNK inhibitor specificity for blocking phospho-JNK1/2 and phospho-c-Jun; B, effect of JNK inhibitor SP600125 or p38MAPK inhibitor SB202190 on apoptosis induction by SN38/MSeA. DU145 cells were preloaded with the inhibitors for 1 hour before they were treated with MSeA and SN38 for 24 hours; *, $P < 0.05$ compared with SN38/MSeA combination treatment ($n = 4$ replicates).

p38MAPK with SB202190 did not protect against apoptosis induced by SN38/MSeA (Fig. 3B, column 6 versus 4). These results suggest that JNK activation played a crucial role in apoptosis induction by SN38/MSeA, whereas p38MAPK activation was not a factor. The potentiation effect of MSeA on SN38-induced apoptosis was not mediated by further increasing SN38-induced JNK activation, but rather by affecting some molecular targets downstream of JNK/c-Jun.

Caspase-Mediated 7-Ethyl-10-Hydroxycamptothecin/Methylseleninic Acid-Induced Apoptosis. Because our earlier work has shown a critical dependence on caspases in apoptosis execution by MSeA in DU145 cells (5), we investigated selected key caspases that might be further amplified by the SN38/MSeA combination. The cleavage activation of the "initiator" caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) and the executioner caspase-3 and its canonical substrate PARP was detected by immunoblot analyses (Fig. 4A). SN38 alone barely induced detectable levels

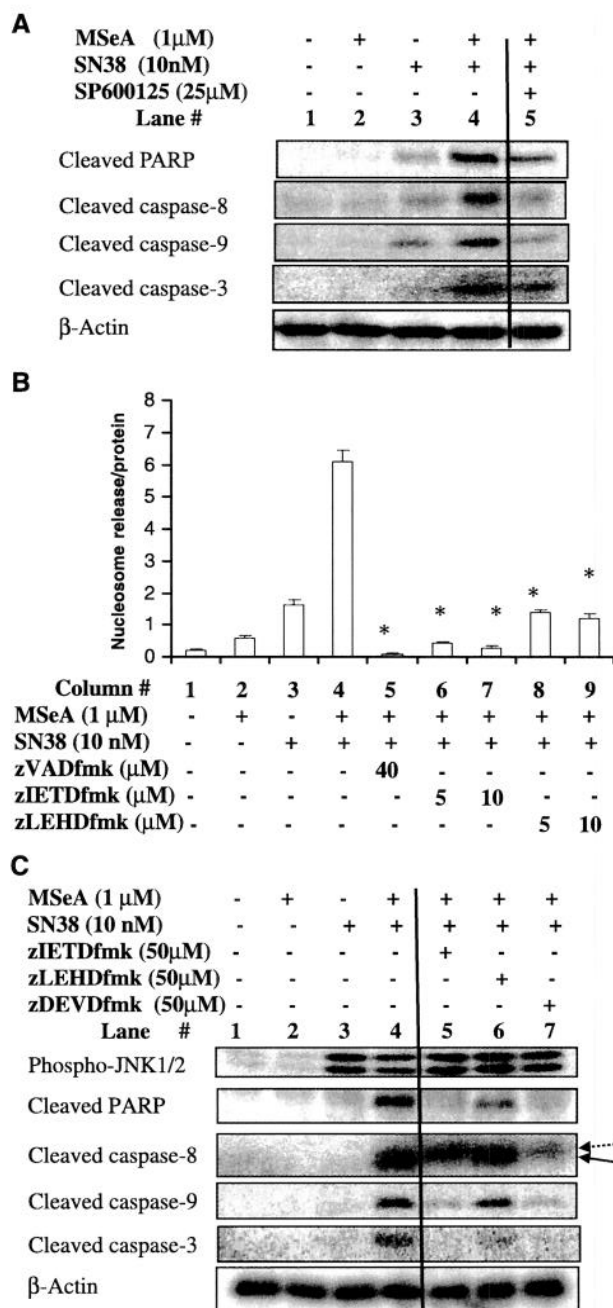


Fig. 4 A, immunoblot detection of cleaved PARP and selected caspases after 24 hours of exposure to MSeA and/or SN38 and the effects of JNK inhibitor SP600125 on these parameters; B, effect of the general caspase inhibitor zVADfmk and the caspase-8- and caspase-9-specific inhibitors on apoptosis induction by SN38/MSeA in DU145 cells; *, $P < 0.05$ compared with SN38/MSeA combination treatment ($n = 4$ replicates); C, immunoblot analyses of effects of caspase inhibitors on JNK1/2 phosphorylation and caspase cleavage after 24 hours of exposure to SN38/MSeA; (solid arrow) fully processed caspase-8; (dashed arrow) migration retarded form, suggesting incomplete processing.

of cleaved caspases and PARP (lane 3), and by the same token, MSeA alone was completely ineffective (lane 2). In contrast, the SN38/MSeA combination markedly increased the cleavage of all three caspases and PARP (lane 4). These results are consistent with apoptosis ELISA data shown in Fig. 3B.

To test the functional significance of the activated caspases and the relative contribution of caspase-8 and caspase-9, we used general and specific caspase inhibitors to interfere with the activity of either total caspases or a particular caspase. The general caspase inhibitor zVADfmk completely repressed apoptosis induced by MSeA/SN38 (Fig. 4B, column 5 versus 4). The caspase-8 inhibitor zIETDfmk blocked apoptosis completely at concentrations as low as 5 μmol/L (columns 6 and 7 versus 4). The caspase-9 inhibitor zLEHDfmk also exerted a substantial protection against MSeA/SN38-induced apoptosis (~80%; columns 8 and 9 versus 4). Immunoblot analysis of cleaved PARP confirmed the complete reversal effect of the caspase-8 inhibitor (Fig. 4C, lane 5 versus 4) and ~80% decrease by the caspase-9 inhibitor (lane 6 versus 4). The fact that even when used at 50 μmol/L, caspase-9 inhibitor did not completely inhibit PARP cleavage, suggested that ~20% of PARP cleavage and apoptosis were independent of caspase-9. In addition, the caspase-8 inhibitor prevented its own processing to the fully cleaved form (solid arrow) as indicated by the slightly retarded migration of the band (dashed arrow) and effectively blocked the cleavage activation of caspase-9 and caspase-3 (lane 5 versus 4). The caspase-9 inhibitor had no effect on its own cleavage or on caspase-8 cleavage, yet efficiently diminished caspase-3 cleavage (lane 6 versus 4). The above observation was consistent with the interpretation of a caspase-8 to caspase-9 functional activation hierarchy to carry out the majority (estimated ~80%) of the caspase signaling from caspase-8 to further downstream caspases, such as caspase-3 and/or -7. As expected of their final executioner role, the caspase-3/-7 inhibitor zDEVDfmk was equally effective as the caspase-8 inhibitor in blocking PARP cleavage (lane 7 versus 5). Paradoxically, zDEVDfmk significantly decreased the cleavage of caspase-8 into the fully cleaved form (solid arrow) and the cleavage of caspase-9 as well as caspase-3 (lane 7). These results raised the possibility of a positive feedback loop from caspase-3 and/or caspase-7 to caspase-8 to amplify caspase-8-initiated cascades.

c-Jun-NH₂-Kinase Activation was Necessary for Enhanced Caspase Activation by 7-Ethyl-10-Hydroxycampothecin/Methylseleninic Acid. As far as the relationship between JNK and caspases was concerned, the JNK inhibitor SP600125 significantly diminished the cleavage of caspases-8, -9, and -3 (Fig. 4A, lane 5 versus 4), an outcome that was concordant with the protective effect of the inhibitor detected by the apoptosis ELISA kit (Fig. 3B) and by sub-G₁ analysis (from ~17% without inhibitor to ~6.5%). None of the three caspase inhibitors affected the JNK1/2 phosphorylation status (Fig. 4C, lanes 5-7 versus lanes 3 and 4), indicating that JNK activation was not the consequence of caspase activation or apoptosis. Collectively, these results support the idea that the enhancement by MSeA of SN38-induced apoptosis was mediated primarily through interactions with JNK-dependent targets, rather than JNK or c-Jun, leading to the augmented activation of caspase-8 and other caspases.

Role of c-Jun-NH₂-Kinase in the Potentiation Effect of Methylseleninic Acid on Apoptosis Induced by Etoposide or Paclitaxel. The above notion of enhancement by MSeA of the apoptosis potency of SN38 through JNK-dependent targets was further tested with etoposide (Fig. 5A and B) and

paclitaxel (Fig. 5C and D) in DU145 cells. For etoposide, the patterns of JNK activation, apoptosis response, and protection by the inhibitors were essentially the same as those for SN38. Briefly, etoposide exposure for 24 hours resulted in a significant increase in JNK1/2 phosphorylation (Fig. 5A, lane 3 versus 1), whereas MSeA exposure alone had no activating effect (lane 2 versus 1). Although MSeA did not enhance JNK1/2 phosphorylation beyond that induced by etoposide alone (lane 4 versus 3), their combination synergistically increased the cleavage of caspase-8 and -9 and PARP (lane 4 versus 3) and apoptotic DNA fragments (Fig. 5B, column 4 versus 3). The JNK inhibitor significantly diminished the cleaved caspases-8 and -9 and PARP (Fig. 5A, lane 5 versus 4) and DNA fragmentation (Fig. 5B, column 5 versus 4) induced by etoposide/MSeA. The caspase-8 inhibitor zIETDfmk blocked completely (column 6 versus 4) and the caspase-9 inhibitor zLEHDfmk exerted a major protection (~80%) against apoptosis induced by MSeA/etoposide (columns 7 versus 4).

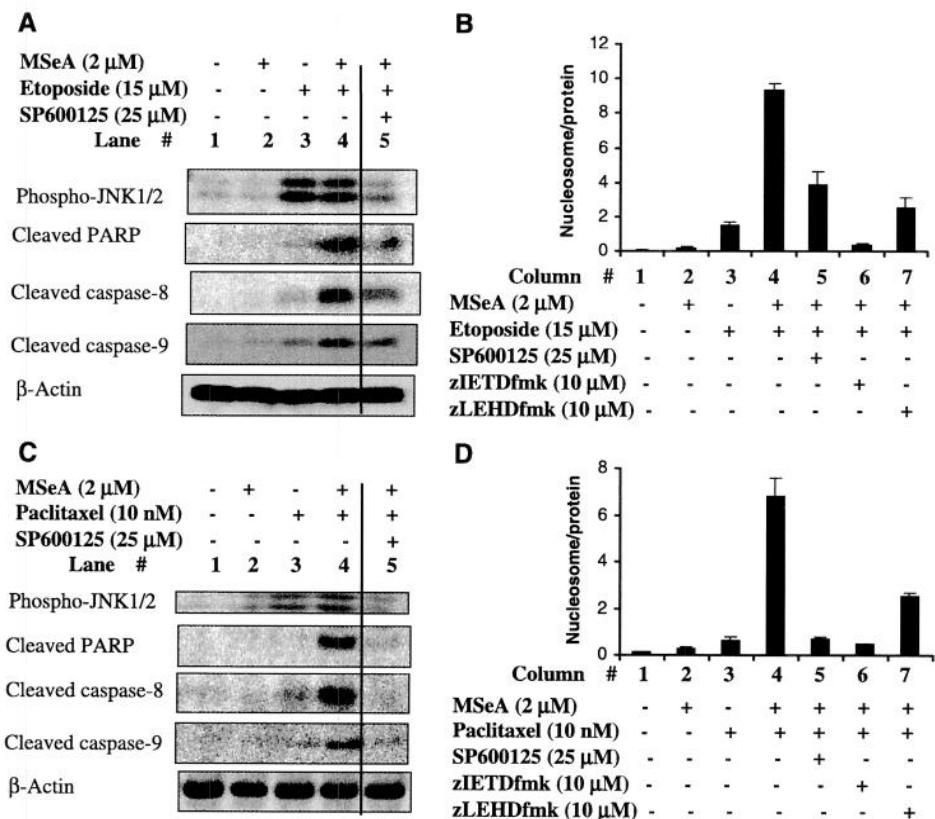
For paclitaxel, treatment for 24 hours increased JNK1/2 phosphorylation (Fig. 5C, lane 3 versus 1), although not as strongly as did the SN38 or etoposide treatment. The paclitaxel/MSeA combination did not enhance JNK1/2 activation beyond the effect of paclitaxel treatment alone (lane 4 versus 3). The JNK inhibitor completely abolished the paclitaxel/MSeA-induced cleavage of caspases-8, -9, and PARP (lane 5 versus 4) and DNA fragmentation (Fig. 5D, column 5 versus 4). The caspase-8 inhibitor abolished, whereas the caspase-9 inhibitor substantially (~60%) decreased DNA fragmentation induced by the MSeA/paclitaxel combination (columns 6 and 7 versus 4).

Taken together, the results of the experiments using all three drugs affirmed the central importance of caspases, especially

caspase-8 as the most likely initiator caspase, in mediating apoptosis induced by the MSeA/drug combination. Because MSeA did not further increase drug-induced JNK activation, interactions between MSeA and JNK-dependent molecular targets must have constituted a major pathway for enhancing caspase activation.

c-Jun-NH₂-Kinase-Independent Suppression of Survival by Topoisomerase Inhibitors. Because the JNK inhibitor did not completely block apoptosis induced by a combination of MSeA with either SN38 or etoposide in DU145 cells (Figs. 4 and 5A and B), we suspected the existence of JNK-independent "targets" for constituting a minor pathway to regulate caspase activation. Inhibitors of apoptosis proteases such as survivin and XIAP bind to caspase-3 to directly inactivate its activity (25). A suppression of the expression of one or more inhibitors of apoptosis proteases might lower the threshold for apoptosis mediated through these caspases. As shown in Fig. 6, SN38 or etoposide exposure alone for 24 hours markedly suppressed survivin expression (Fig. 6A and B versus 3 versus 1), whereas MSeA at the concentrations used had no inhibitory effect (lane 2 versus 1). The combination of MSeA with either SN38 or etoposide did not suppress survivin expression to any greater extent than did each drug alone (lane 4 versus 3). Furthermore, the JNK inhibitor did not restore survivin expression (lane 5 versus 4), supporting a JNK-independent down-regulation of survivin expression by these two drugs. In contrast to SN38 and etoposide, paclitaxel or the paclitaxel/MSeA combination did not affect the expression of survivin (Fig. 6C). In terms of the specificity of suppression of survivin expression by the topoisomerase inhibitors, XIAP expression was not affected by

Fig. 5 Effects of inhibitors of JNK and caspases on apoptosis induced by MSeA in combination with etoposide (A and B) or paclitaxel (C and D) in DU145 cells. A and C, immunoblot detection of JNK1/2 phosphorylation and cleaved PARP and caspase-8 and -9 after 24 hours of exposure to MSeA and/or etoposide (A) or paclitaxel (C) and the effects of JNK inhibitor SP600125 on these parameters; B and D, effects of SP600125 and caspase-8 and -9 inhibitors on apoptosis induced by MSeA/etoposide (B) and MSeA/paclitaxel (D) in DU145 cells. Column, average and SD of four replicates.



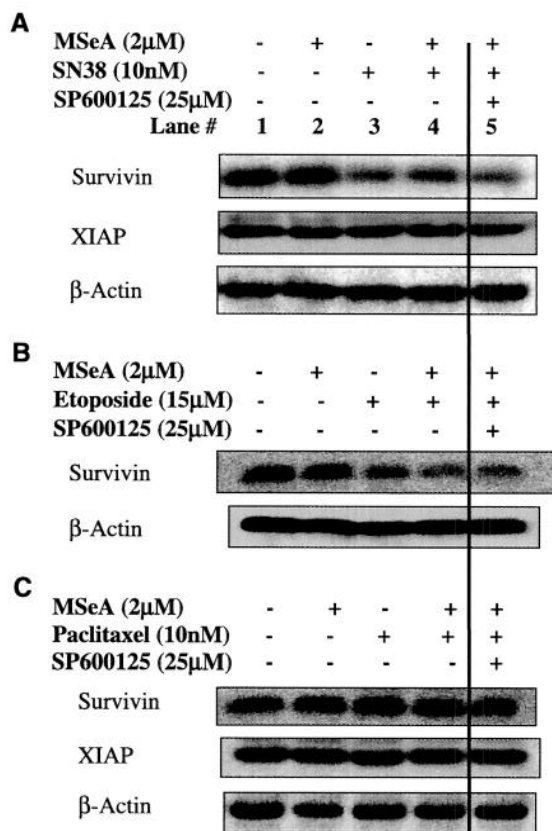


Fig. 6 Immunoblot detection of the endogenous inhibitors of apoptosis proteases survivin and XIAP after 24 hours of exposure to (A) SN38 and/or MSeA; (B) etoposide and/or MSeA; (C) paclitaxel and/or MSeA and the effects of the JNK inhibitor SP600125 on these parameters. β -Actin was probed to indicate the evenness of loading of the protein extract from each treatment.

any of the drugs or their combination with MSeA (Fig. 6A or C). Therefore, these data showed that survivin might be a JNK-independent target for regulation by SN38 and etoposide, possibly contributing to apoptosis signaling that was insensitive to inhibition by the JNK inhibitor.

DISCUSSION

The data reported above document to our knowledge, for the first time, a novel activity of the methylselenol precursor MSeA for enhancing caspase-mediated apoptosis induced by SN38, etoposide, and paclitaxel in two androgen-independent PCA cell lines (Fig. 1). If translatable to *in vivo* studies, our findings have important implications for improving the therapeutic efficacy of these and possibly other chemotherapeutic modalities in patients with advanced metastatic PCA.

Mechanistically, irrespective of the different molecular targeting actions of the three drugs used, our results support not only caspases as key mediators of the augmented apoptosis induced by the combination treatments in DU145 cells but also a compelling role of JNK-dependent downstream targets for interactions with MSeA to amplify caspase activation induced by each drug. As far as caspases are concerned, two well characterized caspase activation pathways mediate apoptosis induced by many chemotherapeutic drugs (26, 27). The mito-

chondrial (intrinsic) pathway generally involves mitochondria permeability transition leading to release of cytochrome *c*. Cytosolic cytochrome *c* then associates with a protein complex known as the apoptosome, leading to activation of caspase-9 which in turn cleaves and activates the effector caspases such as caspases-3 and -7, which mediate the characteristic proteolysis (e.g., PARP cleavage) and DNA digestion. The death receptor (extrinsic) pathway involves the engagement of the death receptors, recruits the adapter protein FADD and procaspase-8, thereby forming a complex known as the death-inducing signaling complex. The consequent proximity of caspase-8 proteins in the death-inducing signaling complex allows their autocleavage and activation. Caspase-8 can directly activate caspase-3 and caspase-7. The apoptotic signal can also be amplified by cross-talk between the two pathways when caspase-8 cleaves Bid, a member of the Bcl-2 family. Truncated Bid translocates to the mitochondria where it facilitates release of cytochrome *c* and the second mitochondrial activator of caspases which binds and inactivates inhibitors of apoptosis proteases, leading to the further activation of caspase-9 and caspase-3. Feedback amplification loops by caspase-3 for enhancing the processing of "initiator" caspases and for mitochondrial release of cytochrome *c* have also been extensively documented in many models (28–30). In particular, with our previous work on MSeA-induced apoptosis in DU145 cells, caspase activity was necessary for mitochondria to release cytochrome *c* (5).

In the current work, MSeA combination with any one of the three drugs resulted in much enhanced activation of caspases-8, -9, and/or -3 and PARP cleavage in DU145 cells, indicative of the involvement of both the extrinsic and intrinsic pathways. With all three drugs, the specific inhibitor for caspase-8 completely blocked apoptosis induced by their combination with MSeA, whereas the caspase-9 inhibitor exerted a major but incomplete protection (ranging from ~60% for paclitaxel to ~80% for SN38 and etoposide; Figs. 4B and C and 5B and D). Based on the caspase cleavage patterns in the presence of caspase inhibitors (Fig. 4C), we propose an activation hierarchy from caspase-8 to other caspases including caspase-9, and caspases-3 and/or -7, and a feedback loop from caspase-3 and/or caspase-7 to caspase-8. The pattern of protection by the specific caspase inhibitors in the current study closely resembled that which we have reported for MSeA-induced apoptosis in DU145 cells (5). This would support a secondary role of the mitochondria and caspase-9 pathway for amplifying the initial activation signal from caspase-8. The specific targets in the extrinsic and intrinsic pathways for MSeA to interact with to achieve the enhancement of caspases in MSeA/drug-exposed PCA cells are currently under investigation.

Our data showed that JNK activation by all three drugs played a critical role for apoptosis signaling upon which MSeA enhanced the activation of caspases in DU145 cells. Because combining MSeA with any of the three drugs did not further increase JNK phosphorylation beyond that induced by each drug alone (Figs. 3A and 5A and C), the data ruled out JNK or *c-Jun* as the proximal targets for MSeA. Furthermore, when apoptosis induced by SN38/MSeA was effectively blocked by caspase inhibitors (Fig. 4B and C), the JNK1/2 phosphorylation level was not affected (Fig. 4C). These results support the notion that MSeA enhances the apoptosis potency of all three drugs

primarily through an interaction with JNK-dependent molecular target(s) induced by these drugs, amplifying the apoptosis signaling to caspase-8 and other caspases. Candidate targets may include death receptors and their ligands (18, 19, 27) or their associated proteins (21, 27) as well as the formation of death-inducing signaling complex. However, we do not rule out the possibility of a direct activating effect of MSeA on the activities of caspases leading to the augmentation of apoptosis. These hypotheses are currently being investigated.

Even though JNK1/2 activation played a major role in MSeA/SN38- or MSeA/etoposide-induced apoptosis, the JNK signaling axis did not fully account for the caspase activation and apoptosis because when JNK activation was abolished by the JNK inhibitor, apoptosis was not completely prevented (~70% reduction; Figs. 3B, 4A, and 5). We believe that the observed suppression of survivin expression by SN38 or etoposide (Fig. 6) may constitute a JNK-independent minor pathway for signaling to caspase-3 and PARP cleavage in addition to the major JNK axis. Consistent with this notion, paclitaxel or paclitaxel/MSeA treatment did not suppress survivin expression (Fig. 6C) and was exclusively dependent on the JNK axis for signaling to the caspases (Fig. 5C and D).

Regarding the selenium chemical specificity of increasing the apoptosis potency of the therapeutic drugs, we observed that selenite did not show any potentiation effect on the three drugs studied here (Fig. 1). In DU145 cells, we have shown that selenite exposure at dose levels higher than those used in the present study induces DNA apoptotic laddering in the absence of PARP cleavage and caspase activities (5, 6). It has been reported that nanomolar to submicromolar concentrations of selenite can inactivate caspase-3 and JNK activity by a direct redox reaction with the reactive cysteinyl sulfhydryls in these enzymes (31, 32). Whether the inactivating action of selenite on both JNK and caspase-3 accounts for the lack of enhancement action on these drugs requires further research.

In addition to these major points, the practical issues of how treatment scheduling might affect the apoptosis potency of the drugs and how much MSeA was necessary to potentiate apoptosis were addressed using SN38 as a prototype agent. The data indicated that the continuous presence of MSeA above a threshold level may be necessary to support its potentiating action (Fig. 2A) and MSeA can amplify death signaling induced by preloaded SN38 independent of any possible effect on drug retention (Fig. 2C). Our results showed that as little as 0.5 $\mu\text{mol/L}$ MSeA was sufficient to enhance the potency of SN38 in DU145 cells (Fig. 2B). For PC3 cells, 5 $\mu\text{mol/L}$ MSeA was necessary to induce a minimal apoptosis response alone and for potentiating apoptosis by cancer drugs (Fig. 1E). As reference values, the average plasma total selenium concentration is 1.5 $\mu\text{mol/L}$ in the study subjects in the clinical trial by Clark et al. (33). Selenium supplementation elevated the concentration to 2.4 $\mu\text{mol/L}$ and reduced the PCA risk by more than half (33). Therefore, the level of selenium enrichment that is needed for enhancing the apoptotic potency of the anticancer drugs studied here may be realistically achievable through supranutritional or pharmacologic supplementation.

In summary, our data support a methylselenium-specific enhancement of the apoptosis potency of three cancer therapeutic drugs through caspase-mediated execution. In spite of their

different mechanisms of action, JNK activation by each of the three drugs seems to be critical for providing the primary death signal and the JNK-dependent targets for interactions with MSeA to amplify the caspase activation cascades. Although the current study dealt with three drugs that activate JNK, it would be interesting and important in the future to determine whether the apoptosis efficacy of other cancer drugs that do not induce JNK can also be enhanced by combination with MSeA or other selenium forms. Such information will help the eventual translation of our observations into clinical benefits for PCA patients.

ACKNOWLEDGMENTS

We thank Todd Schuster, Pat Schmid, and Drs. Guangxun Li for assistance with flow cytometric analyses, and Howard Ganther for generously providing the MSeA used in this study.

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