

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, selenium

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

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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 μL/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.

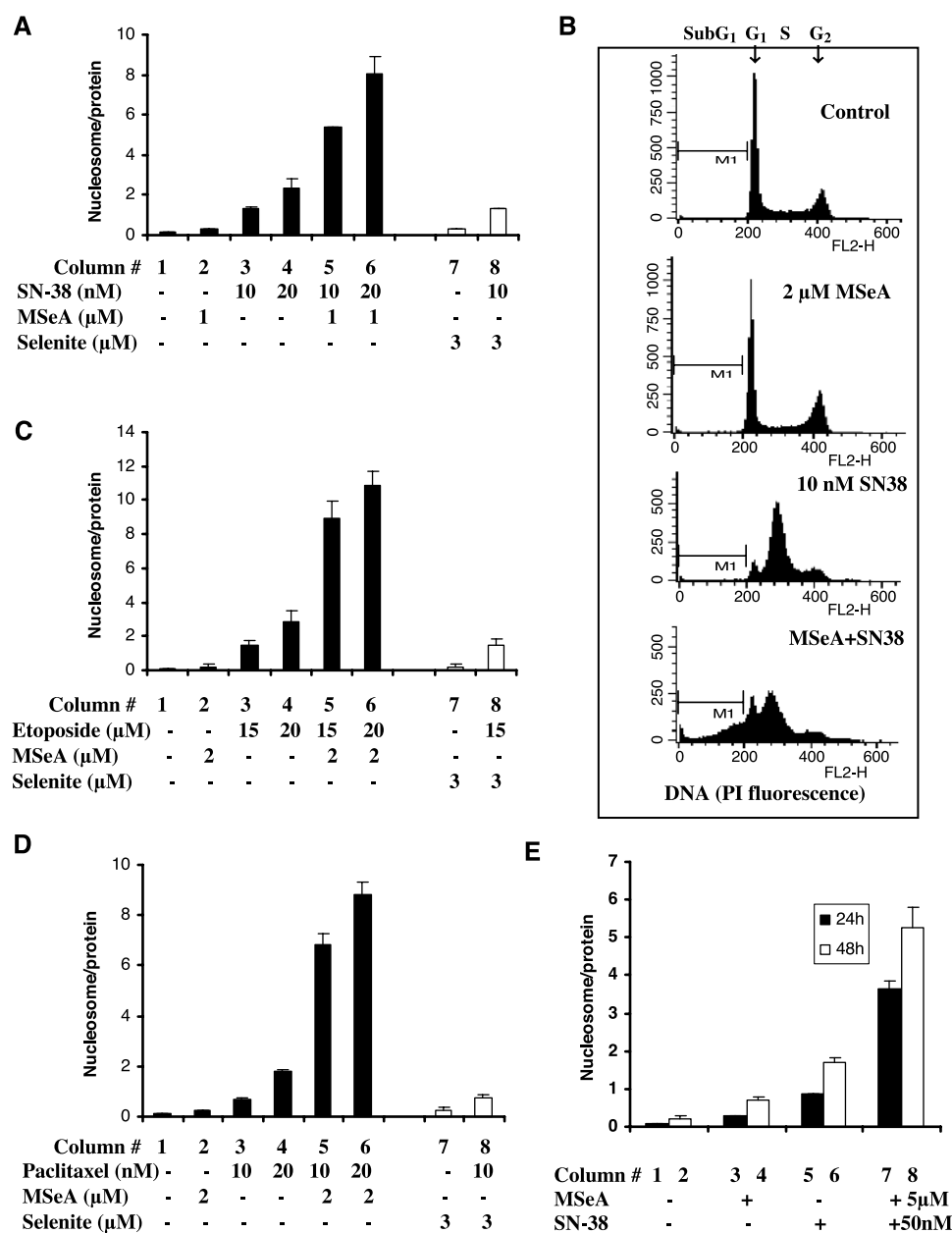
Immunoblot Analyses. Both floating and attached cells were harvested as described above. The cell pellet was washed in PBS twice and the lysate was prepared in radioimmunoprecipitation assay buffer as described previously (5). Immunoblot analyses were essentially as described (5), except that the signals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

RESULTS

Methylselenenic 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 μmol/L MSeA, 10 to 20 nmol/L SN38 or paclitaxel, and 15 to 20 μmol/L etoposide for the combination treatment. As shown in Fig. 1A, exposure to 1 μmol/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 ± SD; n = 3): untreated cells, 1.2 ± 0.4%; cells treated with 2 μmol/L MSeA, 2.6 ± 0.7%; cells treated with 10 nmol/L SN38, 5.7 ± 2.1%; cells treated with the 2 μmol/L MSeA and 10 nmol/L SN38 combination, 16.9 ± 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. 1A, 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 Methylselenenic 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. 2A, 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).

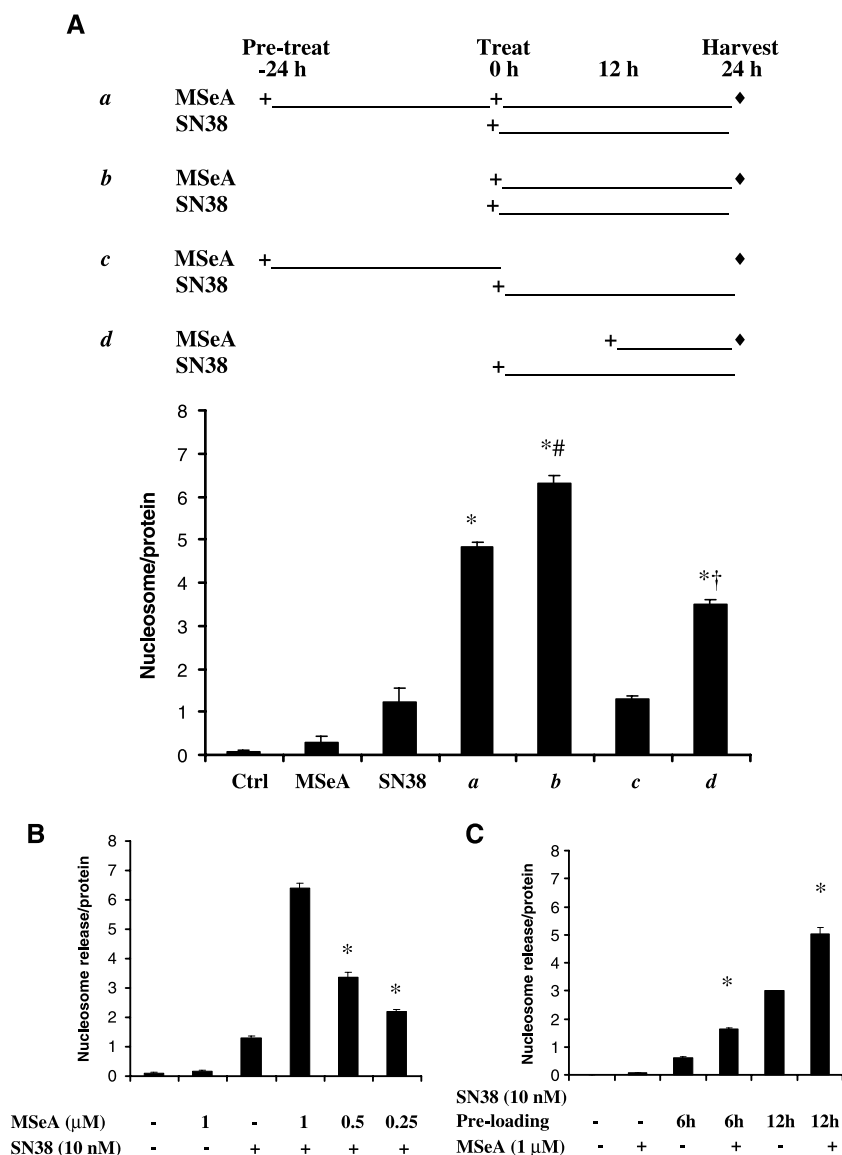


Fig. 2 A, effects of the dosing sequence (regimen) between SN38 and MSeA on the extent of apoptosis in DU145 cells. Dosing regimens (a-d) were as sketched above the plot. Treatment concentration used was 1 $\mu\text{mol/L}$ MSeA and 10 nmol/L SN38, respectively. *, $P < 0.05$ compared with the SN38 treatment 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 was 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

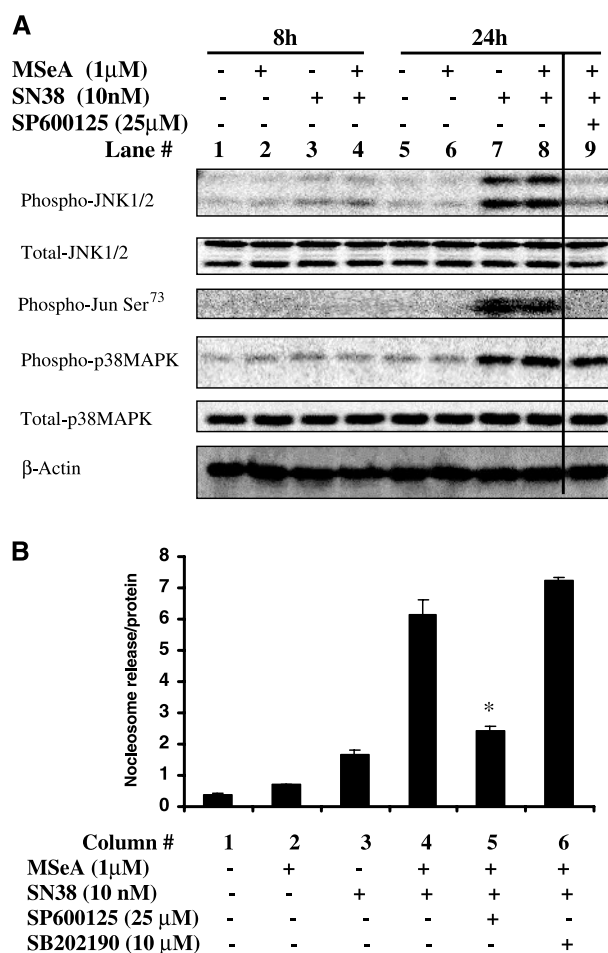


Fig. 3 A, immunoblot detection of phosphorylated JNK1/2, p38MAPK, and *c-Jun* in DU145 cells at 8 or 24 hours of exposure to SN38 and/or MSeA and the verification of JNK inhibitor specificity for blocking phospho-JNK1/2 and phospho-*c-Jun*; B, effect of JNK inhibitor SP600125 or p38MAPK inhibitor SB202190 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

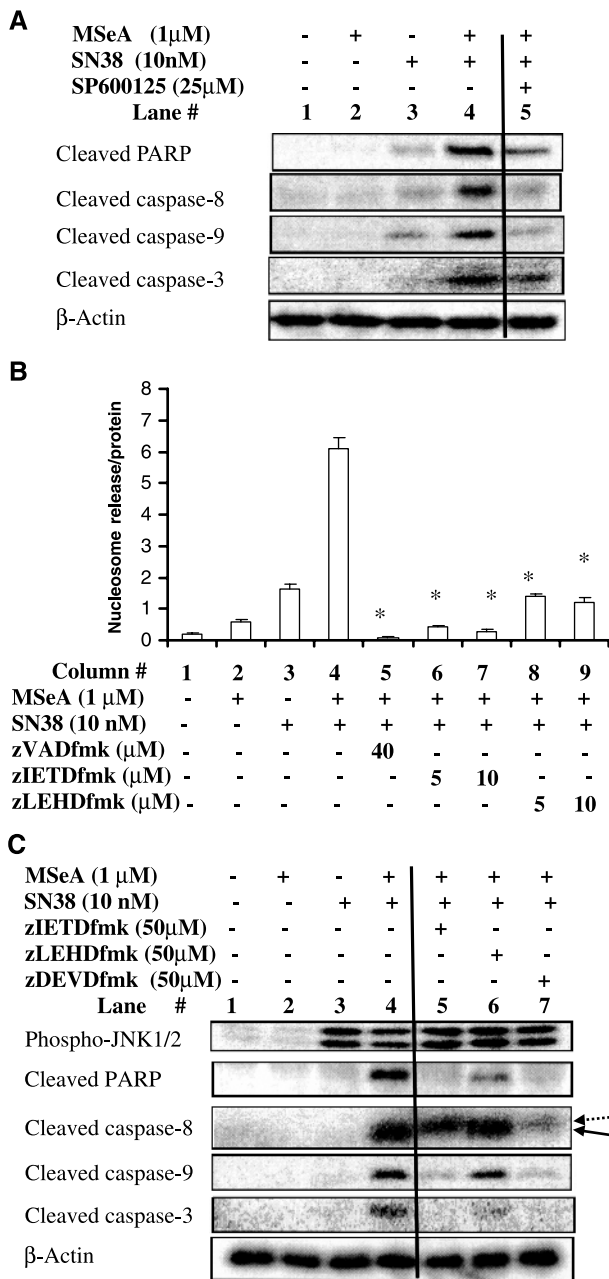


Fig. 4 A, immunoblot detection of cleaved PARP and selected caspases after 24 hours of exposure to MSeA and/or SN38 and the effects of JNK inhibitor SP600125 on these parameters; B, effect of the general caspase inhibitor zVADfmk and the caspase-8- and caspase-9-specific inhibitors on apoptosis induction 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 μ mol/L (columns 6 and 7 versus 4). The caspase-9 inhibitor zLEHDFmk also exerted a substantial protection against MSeA/SN38-induced apoptosis ($\sim 80\%$; 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 $\sim 80\%$ decrease by the caspase-9 inhibitor (lane 6 versus 4). The fact that even when used at 50 μ mol/L, caspase-9 inhibitor did not completely inhibit PARP cleavage, suggested that $\sim 20\%$ 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 $\sim 80\%$) 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-Hydroxycamptothecin/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 $\sim 17\%$ without inhibitor to $\sim 6.5\%$). 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).

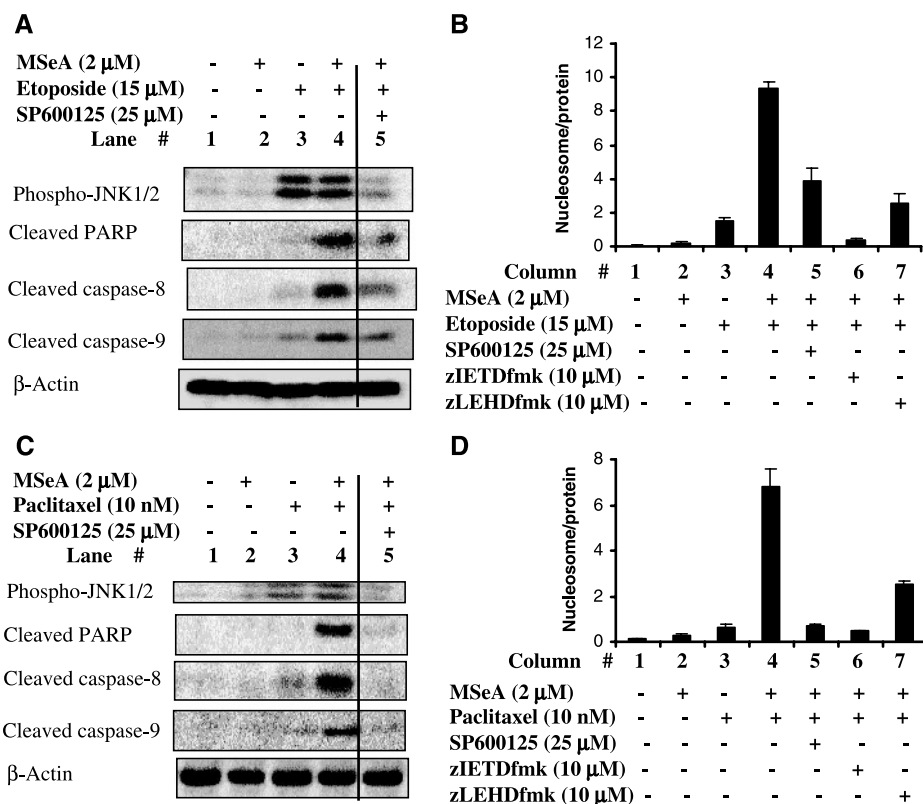
For paclitaxel, treatment for 24 hours increased JNK1/2 phosphorylation (Fig. 5C, lane 3 versus 1), although not as strongly as did the SN38 or etoposide treatment. The paclitaxel/MSeA combination did not enhance JNK1/2 activation beyond the effect of paclitaxel treatment alone (lane 4 versus 3). The 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.



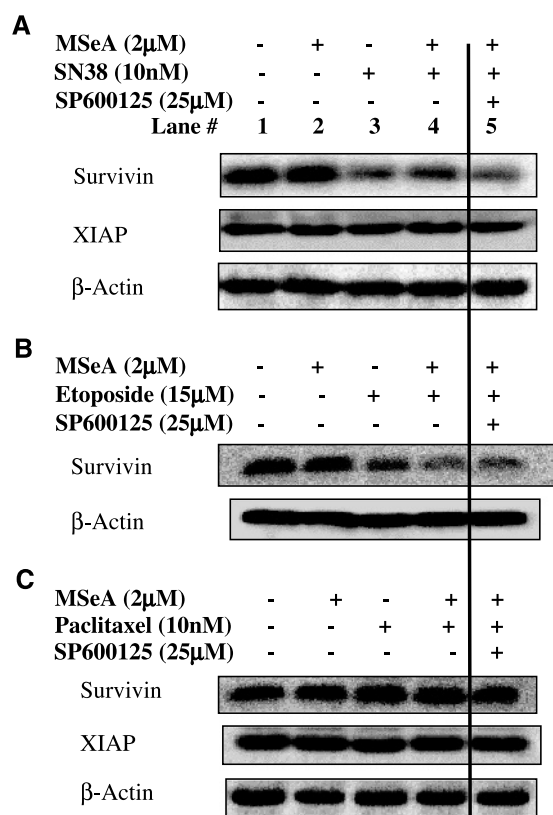


Fig. 6 Immunoblot detection of the endogenous inhibitors of apoptosis proteases survivin and XIAP after 24 hours of exposure to (A) SN38 and/or MSeA; (B) etoposide and/or MSeA; (C) paclitaxel and/or MSeA and the effects of the JNK inhibitor SP600125 on these parameters. β -Actin was probed to indicate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

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