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# Notice of Retraction

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**Re: Yin MB, Li Z-R, Cao S, Durrani FA, Azrak RG, Frank C, and Rustum YM (2004) Enhanced 7-ethyl-10-hydroxycamptothecin (SN-38) lethality by methylselenocysteine is associated with Chk2 phosphorylation at threonin-68 and down-regulation of Cdc6 expression. *Mol Pharmacol* 66:153–160.**

An internal inquiry committee at the Roswell Park Cancer Institute has determined that the above article by Yin et al. (2004) contains substantial sections of text that have been taken verbatim from earlier publications without clear and unambiguous attribution. Although the articles from which the text is taken are cited one or more times in the article, there are several instances in which sections of duplicated text of 10 or more words, many including several consecutive sentences, have been replicated directly without the use of quotation marks or clear citation. Many of these unattributed quotations are from two articles: Blanchard et al. (2002) and Burhans et al. (2002) (references below).

An ad hoc review committee appointed by ASPET's Board of Publications Trustees has reviewed the articles concerned. Generally, but not exclusively, the plagiarized text and appropriation of ideas occur in the Introduction and Discussion sections of the article. There is no allegation that the data reported in the article is unsound, and the main subject of the article is different from the topics of the articles by Blanchard et al. (2002) and Burhans et al. (2002). Nevertheless, the ASPET committee concurs with the Roswell Park internal inquiry committee in its decision that this extent of unattributed textual copying amounts to plagiarism as defined by the U.S. Department of Health and Human Services Office of Research Integrity. The Society considers misappropriation of intellectual property and duplication of text from other authors or publications without clear and unambiguous attribution totally unacceptable. The Society will continue to seek improved ways to identify plagiarized text in the future.

The article has been retracted at the authors' request.

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Articles from which text has been replicated without clear attribution in the article by Yin et al. (2004) include:

Blanchard F, Rusiniak ME, Sharma K, Sun X, Todorov I, Castellano MM, Gutierrez C, Baumann H, and Burhans WC (2002) Targeted destruction of DNA replication protein Cdc6 by cell death pathways in mammals and yeast. *Mol Biol Cell* **13**:1536–1549.

Burhans WC, Blanchard F, and Baumann H (2002) Origin licensing and programmed cell death: a hypothesis. *Cell Death Diff* **9**:870–872.

# Enhanced 7-Ethyl-10-hydroxycamptothecin (SN-38) Lethality by Methylselenocysteine Is Associated with Chk2 Phosphorylation at Threonine-68 and Down-Regulation of Cdc6 Expression

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## ABSTRACT

Methylselenocysteine (MSC) is an organic selenium compound in preventative clinical trials involving prostate, lung, and colon carcinoma. We found that methioninase-activated MSC potentiates 7-ethyl-10-hydroxycamptothecin (SN-38)-induced cell lethality in vitro in the p53-defective human head and neck carcinoma A253 cells. Activated MSC increases chk2 phosphorylation at threonine-68 induced by SN-38, with no significant effect on chk1 phosphorylation. Cell cycle arrest induced by SN-38, however, was not abrogated or potentiated by MSC. These results suggest that the enhanced cellular lethality of SN-38 by MSC was not associated with cell cycle regulation pathways. Because chk2, in addition to its role in cell cycle arrest, can induce apoptosis by phosphorylation/activation, we examined whether increased chk2 phosphorylation could induce preapoptotic DNA fragmentation. DNA damage analysis showed that megabase DNA fragmentation is decreased, accompanied by the increased 30 to 300 kilobase pairs of DNA fragmentation after exposure to SN-38 with MSC,

compared with SN-38 alone. No significant changes in the amount of DNA fragments were observed in cells treated with SN-38 or MSC alone. Moreover, proteolytic destruction of DNA replication-associated proteins cdc6, MCM2, and cdc25A may induce a DNA damage checkpoint response. The observed down-regulation of DNA replication proteins cdc6, MCM2, and cdc25A after exposure to SN-38 with MSC further indicates a relationship between drug response and DNA damage. Exposure to SN-38 with MSC resulted in a significant increase of poly(ADP-ribose) polymerase cleavage and caspase 3 activation. All together, the data support the hypothesis that enhanced lethality of this combination is associated with increased chk2 phosphorylation at threonine-68 and down-regulation of specific DNA replication-associated proteins, which result in poly(ADP-ribose) polymerase cleavage, caspase 3 activation, and the induction of 30 to 300 kilobase pairs of DNA fragmentation.

Selenium is an essential dietary trace element that plays an important role in a number of biological functions. Recent studies on selenium have focused on its chemopreventive activity (Ganther, 1999). Our experimental studies in cell cultures and animal models demonstrated that MSC can potentiate cell growth inhibition in vitro and antitumor activity in vivo, induced by topoisomerase I (topo I) poisons in the p53-defective human head and neck carcinoma A253 cells. Although several mechanisms have been proposed for enhancing SN-38 cytotoxicity by MSC, the effects on abroga-

tion of cell cycle arrest and apoptosis induction are considered the most feasible mechanism (Yu et al., 2002).

The data from our lab first demonstrated that a better prediction of sensitivity to topo I poisons may be obtained by the characterization of chk1 or chk2 cell cycle checkpoint pathways (Yin et al., 2000). A number of references directly linking chk1 inhibition to checkpoint abrogation would be expected (Hirose et al., 2001; Yu et al., 2002; Kohn et al., 2003). Our data also indicate that the chk1 signaling pathways that mediate cell cycle checkpoints are associated with cellular resistance to topo I poisons in the drug-resistant cell line (Yin et al., 2002). All the data have shown that cell cycle checkpoint regulation pathways are associated with drug sensitivity and resistance to topo I poisons. Thus, we propose

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**ABBREVIATIONS:** topo, topoisomerase; SN-38, 7-ethyl-10-hydroxycamptothecin; SRB, sulforhodamine B; MSC, Se methylselenocysteine; METase, L-methionine- $\alpha$ -deamino- $\gamma$ -mercaptomethane lyase (also known as methioninase); PI, propidium iodide; HBSS, Hanks' balanced salt solution; PFGE, pulsed-field gel electrophoresis; TBE, Tris-borate/EDTA; SeMET, seleno-L-methionine; kb, kilobase(s); PARP, poly(ADP-ribose) polymerase; PML, promyelocytic leukemia gene; chk, checkpoint kinase; MCM, minichromosome maintenance.

that the potentiated drug sensitivity observed with MSC administered before SN-38 is associated with alteration of specific biochemical and molecular markers associated with chk1 and chk2 cell cycle regulation pathways.

Recent experiments (Yu et al., 2001; Sordet et al., 2003) indicated that reduced expression of chk2/hcds1 might promote a p53-independent apoptotic response. In addition, antisense inhibition of chk2/hcds1 expression attenuates DNA damage-induced S and G<sub>2</sub> checkpoints and enhances apoptotic activity in human embryonic kidney 293 cells. These recent data provide the evidence that deregulation of chk2/hcds1 in p53-null cells contributes to enhanced apoptosis. These authors also suggest that the DNA-damage regulating kinase chk2, in addition to its role in cell cycle arrest and/or DNA repair, can induce apoptosis by phosphorylation/activation. Thus, chk2 expression and phosphorylation may play an important role in regulating cell death.

Origin licensing proteins cdc6, preRC, and MCM2, play important roles in regulating cell death, in addition to regulating DNA replication (Blanchard et al., 2002; Burhans et al., 2002; Pelizon et al., 2002). These results show that multiple p53-independent, proteasome- and/or caspase-dependent pathways destroy the replication initiation protein cdc6, which is required for the assembly and/or maintenance of preRCs, at an early stage of apoptosis. The proteasome-dependent destruction of cdc6, which was induced by the DNA-damaging drugs, occurs upstream of or parallel to the action of caspases. Thus, the destruction of cdc6 may be a primordial programmed cell death response that uncouples DNA replication from the cell division cycle.

In this study, we report that MSC can potentiate cell lethality induced by topo I poison SN-38. Because SN-38 treatment in combination with MSC resulted in increased chk2 phosphorylation at Thr68 and down-regulation of DNA replication-associated proteins cdc6, MCM2, and cdc25A, accompanied by the induction of preapoptotic DNA fragmentation, we hypothesize that the alterations of chk2 phosphorylation and cdc6 expression were associated with the increased response of A253 cells to SN-38 by MSC.

## Materials and Methods

**Reagents.** SN-38 was kindly supplied by Pharmacia Corporation (Kalamazoo, MI). The compound was dissolved in dimethyl sulfoxide with final dilutions made in the growth medium. MSC was obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K and RNase A were obtained from Roche Diagnostics Corp. (Indianapolis, IN). L-Methioninase (METase) was purchased from Wako Chemical USA, Inc. (Richmond, VA).

The following antibodies were used: anti-chk1 monoclonal antibody (G-4), anti-chk2 polyclonal antibody (H-300), anti-cdc6 monoclonal antibody (180.2), and anti-cdc25A monoclonal antibody (F-6), anti-cdc25C, anti-14-3-3 mono- and polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA); phospho-chk1 (Ser345) antibody, phospho-chk2 (Thr68, Thr387, Thr432, and Ser19) antibody, and cleaved caspase-3 (Asp175) (5A1) rabbit monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA); and anti-human MCM2 monoclonal antibody (MBL Co., LTD, Nagoya, Japan). Anti- $\beta$ -tubulin monoclonal antibody (clone TUB 2.1; Sigma) was used to demonstrate equal protein loading. All MSC treatment schedules included METase at 0.01 units/ml.

**Tissue Culture.** Human head and neck carcinoma cell lines A253 (43-HTB) were purchased from American Type Culture Collection (Manassas, VA) and maintained as a monolayer in RPMI 1640 me-

dium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The doubling time of the cells is 27 h, and all treatments were carried out using exponentially growing cell cultures. The cell lines were free from mycoplasma as tested with the mycoplasma plus PCR primer set (Stratagene, Cedar Creek, TX) every 2 months.

**Assays for Cellular Growth Inhibition.** Cell growth inhibition of A253 cells by MSC with or without METase was estimated using the total protein SRB assay as described elsewhere (Skehan et al., 1990). In brief, 400 cells/well were plated in 96-well plates and preincubated for 24 h at 37°C. These cells were treated with MSC for 24 h, with or without 0.01 U/ml METase, and then incubated in drug-free medium for four cell doubling times after drug exposure. Thereafter, the cells were fixed with 10% trichloroacetic acid and further processed according to the published SRB procedure. The optical density was measured at 570 nm using an automated Biokinetics reader (model EL 340; Bio-Tek Instruments, Winooski, VT).

**Assays for Alive and Dead Cell Number.** The cytotoxic effect of SN-38 with or without MSC was evaluated by counting alive and dead cells. The treatment with MSC for 24 h, followed by 2-h exposure to SN-38, was performed on cells in exponential growth at 37°C. After the SN-38 treatment, the cells were washed and then reincubated with drug-free medium for an additional 24 and 48 h. The attached (alive) or floating (dead) cell numbers were counted in a Coulter counter Z2 Series (Beckman Coulter, Inc., Fullerton, CA).

**Immunoblot and Immunoprecipitation Analysis.** Analysis of protein expression in cell extract was performed by Western blot. The protein concentration was quantified by the Bio-Rad detergent-compatible protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of cell extract was loaded and separated on SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Bio-Rad). Western blot was performed with antibodies listed above, and assessed using the enhanced chemiluminescence reagent kit (Pierce & Warriner, Inc. and Analytical Sciences, Boston, MA). Immunoprecipitation was performed as described elsewhere (Yin et al., 2002).

**Flow Cytometry.** Exponentially growing cells were exposed to MSC with METase (0.01 units/ml) for 24 h, followed by 2-h treatment with SN-38, and then maintained in drug-free medium. At the indicated time intervals (Fig. 4), the cells were harvested. Approximately 10<sup>6</sup> cells were resuspended in 1 ml of modified Krishan buffer with propidium iodide (PI; Molecular Probes, Eugene, OR) (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.37% NP40 and 0.05% mg/ml PI, pH 7.4) and kept on ice protected from light for 30 to 60 min. The cells were pelleted, resuspended in fresh modified Krishan buffer with PI, and filtered. The samples were analyzed for DNA content on a FACScan flow cytometer.

**Preparation of Agarose Plugs.** DNA plugs were prepared as described previously (Yin et al., 2000). Approximately 5 × 10<sup>6</sup> cells were washed using Hanks' balanced salt solution (HBSS) and resuspended in 0.1 ml of HBSS. An equal volume of 2% low-melting-point agarose prepared in HBSS was added at 50°C, and the mixture was poured into the molds immediately. Cells embedded in agarose were digested in 20 volumes of lysis buffer containing 0.5 M EDTA, pH 8.0, 10 mM Tris, 1% Sarkosyl, and 1 mg/ml proteinase K for 24 h at 50°C. After washing in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), the DNA plugs were then incubated for 1 h in Tris/EDTA buffer containing 0.2 mg/ml RNase A. Each plug contained approximately 1 × 10<sup>6</sup> cells. The agarose plugs, which contain purified DNA, were kept in a storage buffer (10 mM Tris-HCl and 50 mM EDTA, pH 8.0) at 4°C and used for electrophoresis.

**Constant-Field Gel Electrophoresis Analysis for DNA Fragmentation.** DNA plugs were inserted into wells of a 1.8% agarose gel, which was prepared in 1× Tris-acetate/EDTA buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3). A conventional horizontal submerged unit was used for electrophoresis in Tris-acetate/EDTA buffer at room temperature. After electrophoresis, gels were stained with ethidium bromide and photographed on a UV transilluminator.



**Pulsed-Field Gel Electrophoresis Analysis for DNA Fragmentation.** Agarose gels (1%) were prepared in 0.5× TBE buffer (75 mM Tris, 25 mM boric acid, and 0.1 mM EDTA, pH 8.9). Electrophoresis was carried out using Hex-A-Field horizontal gel electrophoresis apparatus (Invitrogen, Carlsbad, CA), which contains a hexagonal array of electrodes having a reorientation angle of 120°. The electrophoresis was performed in TBE buffer at 14°C with buffer circulation. The electrophoresis was carried out in one phase: 90-s pulse time, 16-h running time at 170 V with TBE buffer circulation at 14°C. A DNA ladder of 1 kilobase and megabase I DNA standards were used for sizing double-strand DNA from 0.001 to 1.9 megabases. After electrophoresis, gels were stained with ethidium bromide and then photographed on a UV transilluminator.

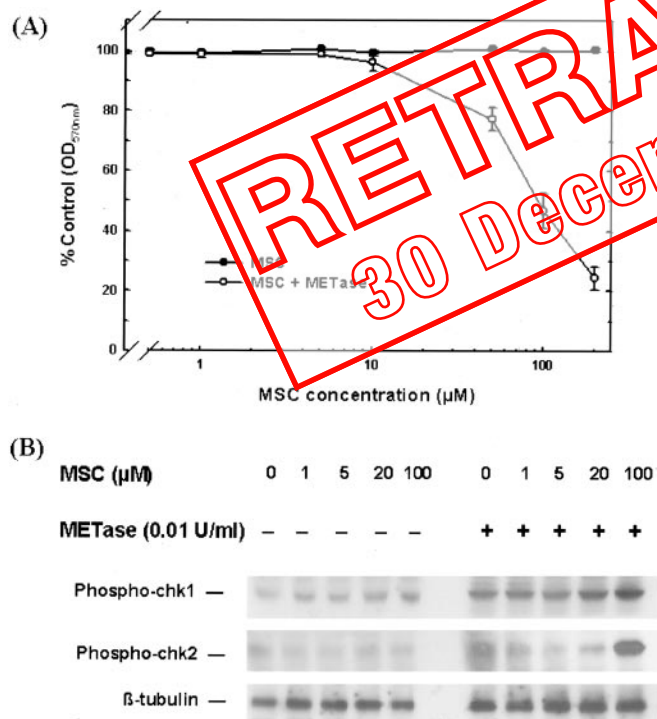
## Results

**Effects of METase on MSC-Induced Cell Growth Inhibition and chk1/chk2 Expression and Phosphorylation.** SeMET cannot be converted to selenol by mammalian cells and is relatively nontoxic, whereas METase catalyzes an  $\alpha,\gamma$ -elimination reaction of SeMET to methylselenol, which damaged the mitochondria via oxidative stress and caused cytochrome *c* release into the cytosol, thereby activating the caspase cascade and apoptosis. The data from Fig. 1A indicate that cellular exposure to MSC alone for 24 h at up to 200  $\mu$ M did not inhibit growth. Activation of MSC by a noncytotoxic concentration of METase (0.01 U/ml), however, yielded a dose-dependent response with  $IC_{10}$  and  $IC_{50}$  values of approximately 20 and 100  $\mu$ M, respectively. In addition,

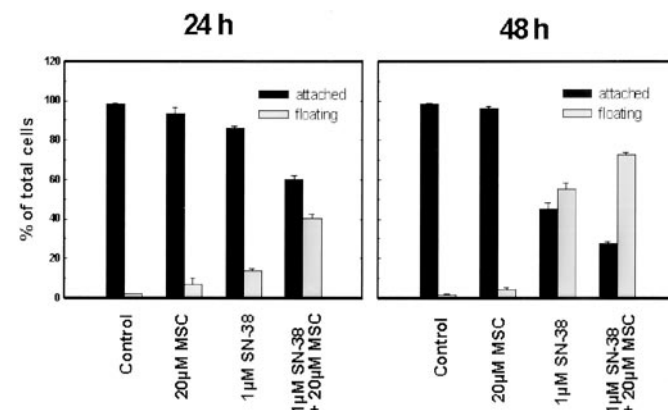
exposure to MSC at up to 100  $\mu$ M in the absence of METase did not induce significant changes in chk1 and chk2 expression or phosphorylation. In contrast, activated MSC induced a moderate increase in chk1 phosphorylation and a significant increase in chk2 phosphorylation in a dose-dependent manner, maximum at 100  $\mu$ M (Fig. 1B). Because 20  $\mu$ M activated MSC was not significantly growth inhibitory and did not significantly alter chk1 or chk2 expression or phosphorylation, this concentration was evaluated as a modulator of SN-38 effects in vitro.

**Effect of Activated MSC on the Viability of A253 Cells Treated with SN-38.** The cytotoxic effect of SN-38 with or without activated MSC were evaluated by counting attached cells, defined as viable, and floating cells, defined as dead or dying cells, at 24 and 48 h after drug exposure. The data shown in Fig. 2 indicate that the viable cells decreased from 86% with SN-38 alone to approximately 60% in the presence of MSC at 24 h and from 45 to 27% at 48 h. Under these same conditions, the dead cells increased from 14% with SN-38 alone to approximately 40% in the presence of MSC at 24 h and from 55 to 73% at 48 h. These results indicate that the decrease in number of viable cells corresponded with an increase in the number of dead cells and hence enhanced the lethality of SN-38 by MSC.

**Chk1 and Chk2 Phosphorylation by SN-38 with MSC.** It has been shown that cell cycle checkpoint regulation pathways are associated with response to topo I poisons. Checkpoint kinases chk1 and/or chk2 regulate S and G<sub>2</sub> arrest. Thus, we postulate that alteration of chk1 or chk2 checkpoint regulation pathways correlates with the observed increased cytotoxic effect of SN-38 by MSC. Analysis of chk1 and chk2 in A253 cells exposed to 1  $\mu$ M SN-38 with or without 20  $\mu$ M activated MSC showed that although exposure to SN-38 with MSC did not significantly induce alteration of chk1 phosphorylation (Fig. 3A), increased chk2 phosphorylation at Thr68 was observed at 24 and 48 h after drug treatment (Fig. 3B). To determine whether SN-38 alone or in combination with MSC induces subsequent autophosphorylation of chk2, phospho-chk2 at Thr387, Thr432, or Ser19 antibodies were used. The results indicate that no significant change in chk2 phosphorylation at Thr387, Thr432, or Ser19 induced by SN-38 with or without MSC was observed (Fig. 3B).



**Fig. 1.** Dose-response curve of MSC with or without METase and effects of METase on MSC-induced chk1 and chk2 phosphorylation. Exponentially growing cells were exposed to MSC with or without METase (0.01 U/ml) for 24 h and then incubated in drug-free medium for four doubling times. Cell growth inhibition was estimated using the total protein SRB assay (A). Exponentially growing cells were exposed to MSC at  $\leq 100$   $\mu$ M with or without METase (0.01 U/ml) for 24 h and then incubated in drug-free medium for an additional 24 h. Cells were harvested and cell extracts were analyzed for chk1 and chk2 phosphorylation by immunoblotting (B).  $\beta$ -Tubulin serves as a loading standard.

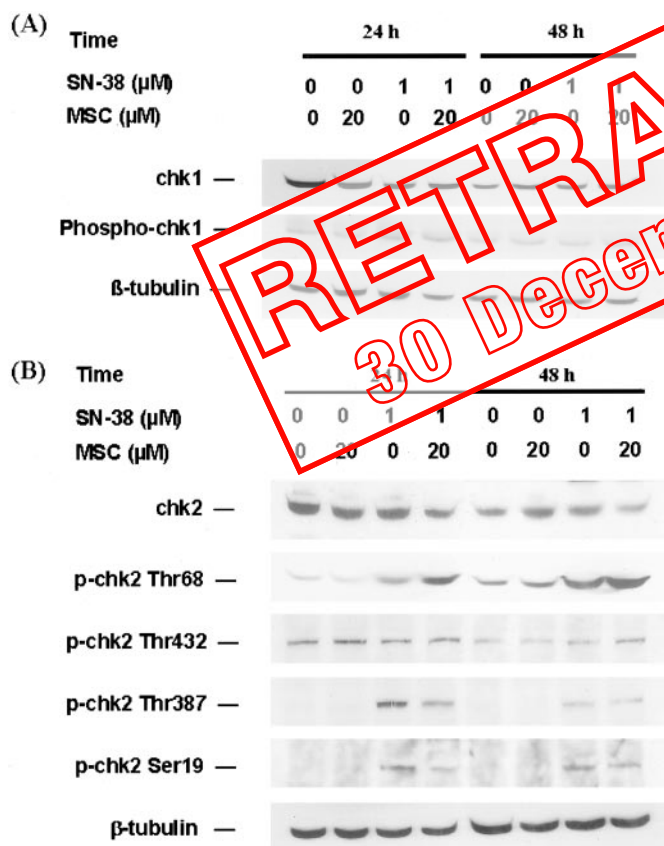


**Fig. 2.** Percentage of viable and dead cells induced by SN-38 with or without MSC. Cells were treated with 20  $\mu$ M MSC for 24 h, followed by 2 h exposure to 1  $\mu$ M SN-38, and then incubated in drug-free medium for 24 and 48 h. The attached cells, defined as viable, and floating cells, defined as dead or dying, were counted in a Coulter counter Z2 Series. Each point corresponds to the mean of at least three experiments.

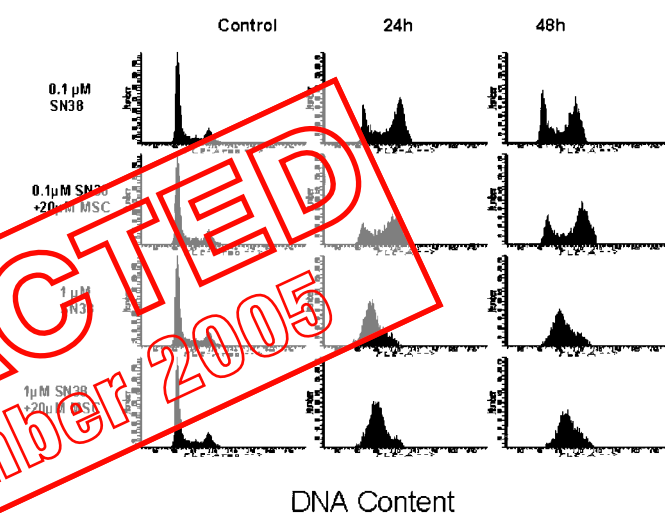
**Cell Cycle Perturbation Induced by SN-38 Alone or in Combination with Activated MSC.** Cell cycle distribution of A253 cells treated for 24 h with MSC (20  $\mu$ M) activated by METase (0.01 U/ml) followed by SN-38 for 2 h were analyzed and the data are summarized in Fig. 4. Although MSC did not alter cell cycle distribution, SN-38-induced alteration of cell cycle distribution was time- and concentration-dependent. Significant accumulation of cells was observed in G<sub>2</sub> phase at 0.1  $\mu$ M SN-38 (IC<sub>50</sub>), and cells were primarily accumulated in S-phase at 1.0  $\mu$ M SN-38 (IC<sub>90</sub>). The data in Fig. 4 also demonstrate that activated MSC did not alter S-phase cell cycle arrest induced by SN-38. Thus, the observed potentiation of cellular growth inhibition of SN-38 by MSC does not seem to be associated with alteration of cell cycle regulation pathways.

**Effect of MSC on SN-38-Induced Alterations of cdc6, MCM2, and cdc25A Expression.** Because an origin licensing protein cdc6 plays an important role in regulating cell death, we next sought to determine whether SN-38 in combination with MSC alters DNA replication-associated protein cdc6 expression. The protein level of cdc6 decreased in response to SN-38 with MSC, compared with SN-38 alone (Fig. 5). To determine whether the decrease in cdc6 levels induced by SN-38 with MSC paralleled other DNA replication-associated proteins, we assessed the expression of MCM2 and

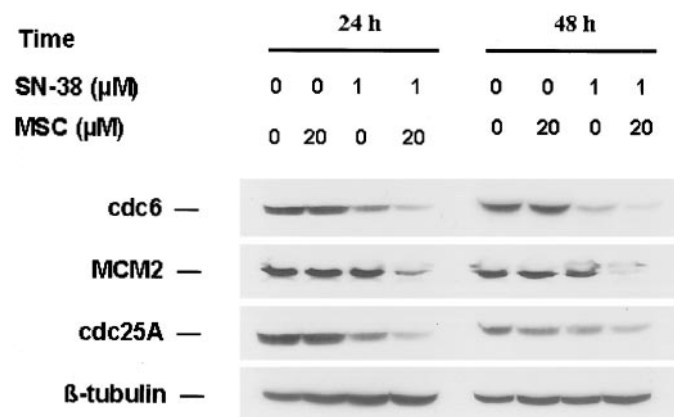
cdc25A. The results indicated that the reduced level of cdc6 protein expression was accompanied by the decrease in MCM2, a protein that interacts in preRCs. In addition, the phosphatase cdc25A, which regulates initiation of DNA replication upstream of the cycle-dependent kinase cdk2, was also degraded in response to SN-38 in combination with MSC. Cdc25C is another chk2 substrate. Our preliminary results indicated although the phosphatase cdc25A was degraded in response to SN-38 in combination with MSC, there are no momentous differences of cdc25C protein expression or 14-3-3 protein binding between SN-38 alone and in combination with MSC (data not shown). These results are consistent with the hypothesis that the destruction of cdc6 occurs as part of a checkpoint that inhibits initiation of DNA replication and requires the destruction of cdc25A. These results infer that the proteolytic destruction of all three proteins (cdc6, MCM2, and cdc25A) is part of a DNA damage



**Fig. 3.** Analysis of the effects of MSC on SN-38-induced chk1 and chk2 expression and phosphorylation. Cell treatment is as described in Fig. 2. At the indicated times, cells were harvested and 50  $\mu$ g of total cell extracts were separated in SDS-polyacrylamide gel electrophoresis. Protein bands were detected with antibodies to total chk1, phospho-chk1, total chk2, and phospho-chk2 at Thr68, Thr387, Thr432, and Ser19.  $\beta$ -Tubulin serves as a loading standard. Blots were representative of results from three separate experiments.



**Fig. 4.** The influence of MSC on cell cycle arrest induced by SN-38. Shown are representative flow cytometry DNA histograms of A253 cells treated with 20  $\mu$ M MSC for 24 h followed by 2-h exposure to 0.1  $\mu$ M and 1  $\mu$ M SN-38. At harvest, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. Abscissa values are proportional stained DNA content and ordinate values indicate the relative numbers of cell cycle distribution. Experiments were performed at least three times, each in duplicate.



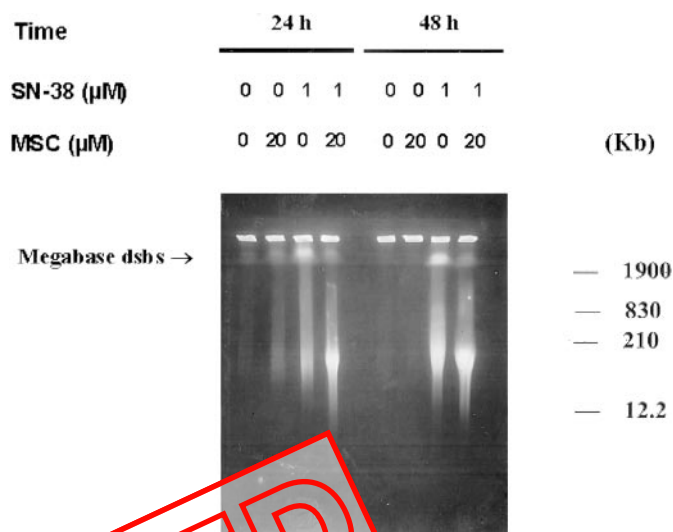
**Fig. 5.** Effect of SN-38 with or without MSC on cdc6, MCM2, and cdc25A protein expression. Cells were exposed to the drug with or without the modulator as described in the legend of Fig. 2 and harvested at 24 and 48 h after drug exposure. Cell extracts were analyzed for cdc6, MCM2, and cdc25A by immunoblotting.

checkpoint response that inhibits initiation and occurs independently of the type of DNA lesion.

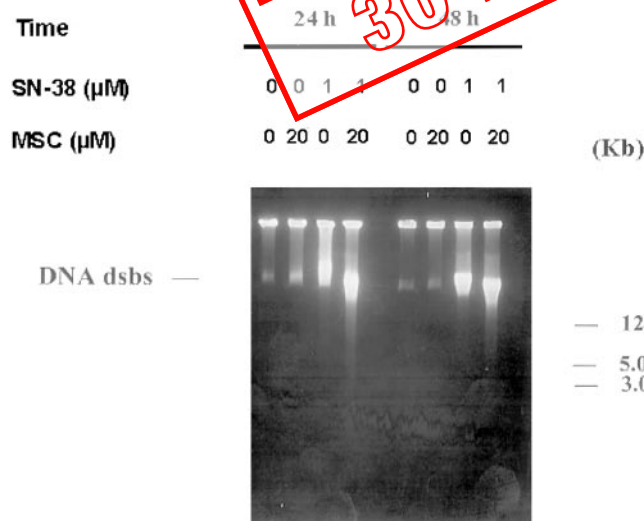
**Analysis for DNA Fragmentation Induced by SN-38 Alone or in Combination with Activated MSC.** The DNA-damage regulating kinase chk2, in addition to its role in cell cycle arrest and/or DNA repair, can induce apoptosis by phosphorylation/activation. Thus, in addition to regulating cell cycle checkpoint pathways, chk2 expression and phosphorylation play an important role in regulating cell death. To investigate the role of chk2 in cell death, we further studied whether the potentiation of SN-38 sensitivity by MSC is associated with the phosphorylation of the DNA-damage regulating kinase chk2 in A253 cells, which resulted in the induction of preapoptotic DNA fragmentation. To assess the patterns of DNA fragmentation induced by SN-38 alone or in combination with activated MSC, the amounts and sizes of DNA fragments were analyzed by constant-field gel electrophoresis and PFGE. Therefore, we asked whether cdc6 destruction by SN-38 with MSC occurs in association with the induction of apoptotic DNA fragmentation. The results in Fig. 7 show that megabase DNA fragmentation was decreased at 24 and 48 h after exposure to SN-38 with MSC, accompanied by the increased 30- to 300-kb DNA fragmentation, compared with treatment with SN-38 alone, although no significant changes in the amounts of DNA fragments were observed with SN-38 alone or with MSC (Fig. 6). This is consistent with previous reports suggesting that the appearance of higher molecular weight DNA fragmentation ranging between 30 and 300 kb was typically associated with apoptotic cell death (Oberhammer et al., 1993; Collins et al., 1997).

**Activation of Caspase 3 and Subsequent Cleavage of Poly-(ADP-ribose)-Polymerase (PARP).** Activation of caspase 3 and cleaved PARP was determined in A253 cells exposed to SN-38 with or without MSC as shown in Fig. 8. The results indicate that an increase in PARP cleavage and caspase 3 activation could be detected beginning at 24 h after exposure to 1  $\mu$ M SN-38 with MSC. No significant PARP

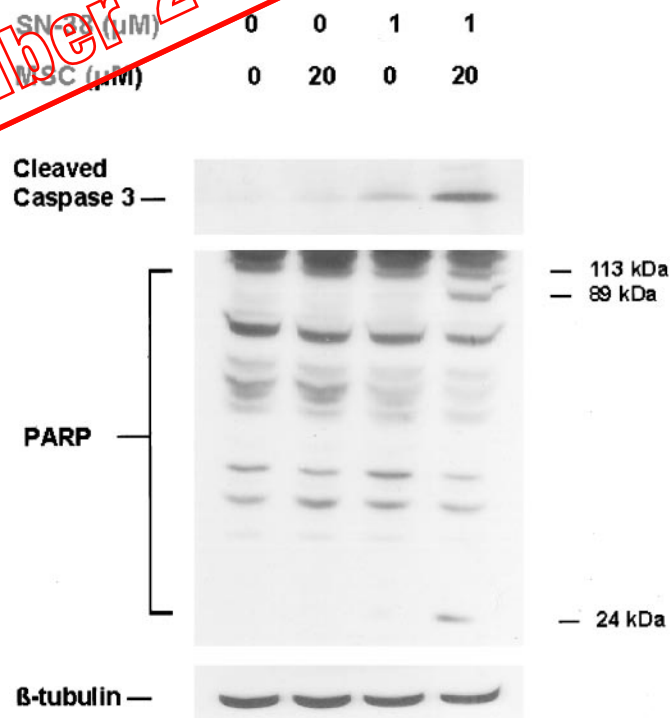
cleavage could be detected at 24 h after treatment with SN-38 alone. An increase in PARP cleavage after drug treatment is expected to induce caspase-mediated apoptosis, which enhances cytotoxicity, thus providing a potential tumor-targeted therapy. These results indicate that at least in this cell line, activation of caspase 3 and subsequent cleavage



**Fig. 7.** PFGE analysis for patterns of DNA fragmentation induced by SN-38 with or without MSC. Agarose plugs used in Fig. 6 also used for PFGE. Determination of DNA damage by pulsed-field gel electrophoresis was carried out in one phase: 80-s pulse time, 16-h running time at 170 V at 1°C in 1% agarose. DNA ladder of 1 kb and megabase I DNA standards are suitable for sized double-stranded DNA from 0.001 to 1.9 megabases.



**Fig. 6.** Kinetics of DNA double-strand breaks induced by SN-38 with or without MSC. Exponentially growing cells were treated with MSC for 24 h, followed by 2-h exposure to 1  $\mu$ M SN-38, and then incubated in drug-free medium for an additional 24 or 48 h. Cells were collected and determination of DNA fragmentation was performed using constant-field gel electrophoresis with the agarose plugs as described under *Materials and Methods*.



**Fig. 8.** Cleaved caspase 3 and PARP cleavage in the A253 cells exposed to SN-38 with or without MSC. Cells were treated, harvested at 24 h after drug exposure, and cell lysates for Western blot analysis were prepared as described in Fig. 2.  $\beta$ -Tubulin is a loading control. Dashes to the right indicate the size in kilodaltons of the unprocessed and cleaved forms of PARP. Data are representative of experiments performed at least three times.



of PARP may be related to the destruction of licensing proteins and/or increased chk2 phosphorylation.

## Discussion

Selenium is a potent chemopreventive agent in preclinical model systems, and accumulating evidence indicates that selenium also protects against the development of cancer in the human population (Medina and Morrison, 1988; Clark et al., 1996; Ganther, 1999; Reid et al., 2002). Both organic and inorganic forms of selenium have been used, but several organic selenocompounds have shown chemopreventive activities with fewer side effects than selenite (Ip et al., 1994; Sinha et al., 1996; Patterson and Levander, 1997). It has been reported that SeMET cannot be converted to selenol by mammalian cells and is relatively nontoxic (Spallholz, 1994; Stewart et al., 1999), whereas METase catalyzes an  $\alpha,\gamma$ -elimination reaction of SeMET to methylselenol, which damaged the mitochondria via oxidative stress and caused cytochrome *c* release into the cytosol, thereby activating the caspase cascade and apoptosis. Thus, we have identified, using monomethyl selenium compounds that are putative precursors of methylselenol, several cellular and gene-expression responses. Figure 1 indicates that exposure to MSC at up to 100  $\mu$ M in the absence of METase did not induce significant changes in lethality of SN-38. In contrast, METase-activated MSC significantly induced cell lethality in human head and neck carcinoma A253 cells. Because 20  $\mu$ M activated MSC did not significantly alter cell lethality, this concentration was evaluated as a modulator of SN-38 effects in vitro.

Previous studies demonstrated that sensitivity to topoisomerase I poisons is associated with alterations of chk1 or chk2 cell cycle checkpoint pathways (Yin et al., 2000). Phosphorylation of chk1 at serine 345 is associated with the response to topoisomerase I poison SN-38. In addition, the chk1 signaling pathways that mediate cell cycle checkpoint are associated with cellular resistance to topoisomerase I poisons in the drug-resistant cell line (Yin et al., 2001, 2003; Hapke et al., 2002). The published results by others indicated that inhibition of cell cycle checkpoint kinases chk1 and chk2 selectively sensitizes cells that have a disrupted p53 pathway (Hirose et al., 2001; Eastman et al., 2002; Yu et al., 2002; Kohn et al., 2003). Thus, we propose that the potentiated drug sensitivity observed with MSC administered before SN-38 may be associated with alterations of chk1 or chk2 checkpoint regulation pathways.

The experiments presented in this study were designed to examine whether the observed potentiation of in vitro cytotoxic effect of SN-38 by MSC is associated with alterations of checkpoint kinases chk1 and/or chk2 expression and phosphorylation. Our results indicate that although exposure to SN-38 with MSC did not significantly induce alteration of chk1 expression and phosphorylation, a significant increase in chk2 phosphorylation at Thr68 was observed at 24 and 48 h after drug treatment. We also investigated phospho-chk2 at Thr387, Thr432, and Ser19 expression to determine whether SN-38 alone or in combination with MSC induces subsequent autophosphorylation of chk2. The results indicate that no significant changes in chk2 phosphorylation at Thr387, Thr432, and Ser19 induced by SN-38 with MSC were observed. The results further indicate a relationship between a significant increase in chk2 phosphorylation at Thr68 and

the observed potentiation of SN-38 lethality by MSC. Therefore, we suggest that SN-38 treatment with MSC potentiates drug sensitivity through the alteration of DNA damage-induced S and G<sub>2</sub>/M arrest in p53-null cells. However, although exposure to SN-38 with activated MSC increases chk2 phosphorylation at Thr68, we did not expect the drug combination to alter S or G<sub>2</sub> cell cycle arrest. Our results indicated that MSC sensitization of SN-38-induced cytotoxicity does not involve the cell cycle regulation pathways.

Chk2 is a DNA damage checkpoint kinase that is considered to be a tumor suppressor, in that it is mutated in some cancers and in variant forms of the multicancer predisposition syndrome, Li-Fraumeni (Bell et al., 1999; Wu et al., 2000). Published data have shown that down-regulation of chk2 in cells with defective p53 function causes a deficient G<sub>2</sub> checkpoint and promotes apoptosis in a p53-independent manner (Xu et al., 2001; Yu et al., 2001, 2002; Hirao et al., 2002; Yang et al., 2002). Additional published observations show that chk2 is activated in an ATM-dependent manner and induces either cell cycle arrest or apoptosis. Chk2 autophosphorylation is required for full kinase activation after DNA damage, suggesting that autophosphorylation is critical for chk2 function after DNA damage (Wu and Chen, 2003). Furthermore, although chk2 is thought to induce apoptosis by phosphorylating p53 and activating p53-dependent apoptotic pathways after DNA damage, chk2-induced apoptosis also occurs through p53-independent mechanisms (Yu et al., 2001; Yang et al., 2002).

Studies indicate that the promyelocytic leukemia gene (PML) was involved in signaling mechanisms for p53-independent apoptosis (Wang et al., 1998; Yang et al., 2002). By demonstrating the direct effect of chk2-mediated phosphorylation of PML on p53-independent apoptotic signaling after DNA damage, a novel relationship between chk2 and PML in tumor suppression has been established. This indicated that the DNA-damage regulating kinase chk2 could induce p53-independent apoptosis by PML phosphorylation. Thus, we suggest that MSC induces the alteration of chk2 phosphorylation at Thr68 in the A253 cells, probably through the PML pathway, which might promote a p53-independent apoptotic response.

A number of publications have indicated that, in addition to regulating DNA replication, origin licensing (*cdc6*, preRC, and MCM2, 3) plays an important role in regulating cell death (Blanchard et al., 2002; Pelizon et al., 2002). Blanchard et al. (2002) reported that *cdc6* destruction by adozelesin occurs in association with an apoptotic response that results in DNA laddering and includes a caspase inhibitor-sensitive component. Within 4 to 6 h of adozelesin treatment, DNA laddering was apparent in MDA cells. Moreover, adozelesin-induced DNA laddering was significantly reduced by treating MDA cells with the caspase inhibitor I. Therefore, adozelesin induces a caspase-dependent apoptotic response in MDA cells that occurs coincidentally with the destruction of *cdc6*. This suggests that the p53-independent destruction of *cdc6*, which was induced by the DNA-damaging drugs, occurs upstream of or parallel to the action of caspases. Thus, the destruction of *cdc6* may be a primordial programmed cell death response. Another possibility is that the destruction of *cdc6* deregulates the cell cycle during programmed cell death by activating cyclin-dependent kinases at the same time that it disrupts functional origin licensing complexes in cells with

unreplicated or incompletely replicated chromosomes (Saha et al., 1998). Several studies have also detected physical associations between components of preRCs and Rb and/or E2F, both of which have been implicated in DNA damage response and apoptosis in addition to their roles in regulating proliferation (Stern et al., 1998). All of these findings suggest that cdc6 and other components of preRCs form a nexus for cell cycle regulation in which DNA replication is coordinated with mitosis downstream of signaling pathways that regulate cell proliferation, DNA damage response, and cell death.

Cdc6 destruction is also accompanied by the destruction of other cell cycle regulatory proteins, including cdc25A, which regulates initiation of DNA replication upstream of the cycle-dependent kinase cdk2. Cdc25A was destroyed in response to DNA damage as a part of multiple checkpoints that inhibit DNA replication (Agami and Bernards, 2000; Mailand et al., 2000; Falck et al., 2001). Our results also indicated that cdc25A was degraded in response to SN-38 in combination with MSC. This suggests that the destruction of cdc6 and cdc25A occurs as part of a DNA damage checkpoint in response to DNA-damaging agents.

These results have important implications for understanding how DNA damaging agents might exert their cytotoxic and antitumor effects, particularly in cells with defective p53. DNA damage response, under our experimental conditions, is related to the destruction of licensing proteins in these cells. In addition, many DNA-damaging drugs induce similar cell cycle-specific and proliferation-dependent lethal effects in mammalian cells as part of a programmed cell death response to irreparable DNA damage (Bhuyan et al., 1992; Tannock and Hill, 1998). Thus, the results may help to explain some aspects of a relationship between the observed potentiation of SN-38 lethality by MSC and DNA fragmentation. A DNA ladder (molecules of approximately 180–300 base pairs) in agarose gels is known as a major biochemical hallmark of apoptosis. To assess the relationship between the increased ratio of the dead or dying cells and the induction of apoptosis, we analyzed the time course of SN-38-induced DNA laddering. No apparent ladder-type DNA fragmentation induced by SN-38 with or without MSC was observed in the A253 cells (data not shown). However, we used pulsed-field gel electrophoresis to measure DNA fragmentation profiles induced by SN-38 with or without MSC and found that megabase DNA fragmentation decreased at 24 and 48 h after exposure to SN-38 with MSC, accompanied by the increased 30- to 300-kb DNA fragmentation, compared with treatment with SN-38 alone. This is consistent with previous reports suggesting that the appearance of higher molecular weight DNA fragmentation ranging between 30 and 300 kb was typically associated with apoptotic cell death (Oberhammer et al., 1993; Collins et al., 1997).

MSC sensitization of SN-38-induced lethality is probably caused by altered chk2 phosphorylation at Thr68 and down-regulation of cdc6, MCM2, and cdc25A expression, which may induce 30- to 300-kb DNA fragmentation. Accompanied by the induction of 30- to 300-kb DNA fragmentation, increased cleavage of PARP and caspase 3 activation could be detected after exposure to SN-38 with MSC, leading to increased apoptotic cell death. The ability to detect these parameters provides a potential tumor-targeted therapy. Confirming the in vitro molecular alterations induced by SN-38

in combination with MSC and the selective modulation seen with the combination of irinotecan and selenium in vivo should provide the basis for validation of these results clinically.

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