

# Activity of Se-Allylselenocysteine in the Presence of Methionine $\gamma$ -Lyase on Cell Growth, DNA Integrity, Apoptosis, and Cell-Cycle Regulatory Molecules

Zongjian Zhu,<sup>1</sup> Weiqin Jiang,<sup>1</sup> Howard E. Ganther,<sup>2</sup> Clement Ip,<sup>3</sup> and Henry J. Thompson<sup>1\*</sup>

<sup>1</sup>Center for Nutrition in the Prevention of Disease, AMC Cancer Research Center, Denver, Colorado

<sup>2</sup>Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin

<sup>3</sup>Department of Experimental Pathology, Roswell Park Cancer Institute, Buffalo, New York

Se-allylselenocysteine (ASC) is effective in inhibiting mammary epithelial cell growth in vitro and mammary carcinogenesis in vivo, but its mechanism is unknown. We recently reported that ASC reduces cell growth in a dose- and time-dependent manner, induces a loss of DNA integrity, and increases apoptosis. However, the level of ASC required for growth inhibition in vitro is 10- to 20-fold higher than that required in vivo. One possible explanation for this difference is that the cells used in in vitro studies have limited lyase activity required to release the allyl Se moiety from selenocysteine, whereas animals have abundant lyase activity in tissues. In the present study, we found that methionine  $\gamma$ -lyase (MGL) added to culture medium containing ASC produced biological effects with lower levels of ASC, comparable to the selenium levels in plasma achieved during in vivo chemoprevention. The combination of 2.5  $\mu$ M ASC and MGL inhibited the growth of TM12 cells and increased apoptosis without loss of DNA integrity. Treatment of TM12 cells with ASC and MGL resulted in an elevation of the protein levels of p53, Cip1/p21, and Kip1/p27, concomitant with a decrease in cyclins D1 and E and modest reductions in cyclin-dependent kinase inhibitors 4 and 2. Cells treated with ASC and MGL also showed decreased phosphorylation of retinoblastoma tumor-suppressor protein. Taken together, these results suggest that a physiologically relevant concentration of ASC with MGL exerts an inhibitory effect on cell growth and that this effect is likely to involve modulation of signaling pathways that suppress the phosphorylation of retinoblastoma tumor-suppressor protein. *Mol. Carcinog.* 29:191–197, 2000.

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## INTRODUCTION

Selenium exerts chemopreventive activity against various types of cancer in numerous animal model systems [1–4]. The biologic activity of Se is not represented by the element per se but is an expression of Se in a variety of chemical forms [1]. Accumulating evidence from both in vitro and in vivo studies has pointed to a monomethylated form of Se as the critical metabolite involved in mediating the growth inhibition of cancer cells [1,2]. Se-methylselenocysteine (MSC), a stable selenoamino acid that is capable of generating methylselenol endogenously through the action of  $\beta$ -lyase or related lyases in a facile one-step reaction, has been shown to be an excellent chemopreventive agent [5].

To explore whether modification of the aliphatic side chain of a selenoamino acid could improve its cancer-inhibitory activity, Se-allylselenocysteine (ASC) was developed and found to be more active than MSC in protecting against chemically induced mammary carcinogenesis in the rat [6]. To assess the

role of cytotoxicity in chemoprevention by ASC, the investigation was extended to characterize the effects of ASC on cell growth, DNA integrity, and apoptosis with the use of an in vitro model [7]. The results showed that ASC reduces the number of adherent cells in a mouse hyperplastic mammary epithelial cell culture. The response is more prominent in p53 wild-type cells than in p53 mutant cells. ASC also increases apoptosis and decreases DNA integrity of these cells. However, additional in vivo data obtained from the rat mammary gland indi-

\*Correspondence to: AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214.

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Abbreviations: MSC, Se-methylselenocysteine; ASC, Se-allylselenocysteine; MGL, methionine  $\gamma$ -lyase; CDK, cyclin-dependent kinase; Rb, retinoblastoma tumor-suppressor protein; PBS, phosphate-buffered saline.

cated that there is no loss of DNA integrity at chemopreventive doses of ASC that result in a plasma concentration of about 6  $\mu\text{M}$  [7]. This level is approximately 12% of the 50- $\mu\text{M}$  concentration of ASC used in these cell-culture experiments.

The  $\beta$ -lyase enzyme has been found in the cytosol of several rat tissues, and the activity is higher in kidney and liver than in intestine and mammary gland [6]. Cultured mouse hyperplastic mammary epithelial cells also express  $\beta$ -lyase, but the activity is relatively low [7]. Differences in the ability to generate allylselenol through lyase enzyme activity may explain the difference in the amount of ASC required to achieve inhibitory effects *in vivo* versus *in vitro*. To test this hypothesis, we used methionine  $\gamma$ -lyase (MGL; EC 4.4.1.11) in our *in vitro* model to enhance the rate of allylselenol release from ASC. This enzyme has been shown to catalyze an Se-alkyl elimination reaction with selenomethionine, methionine, and S-alkylated cysteine derivatives [8]. It catalyzes  $\alpha,\gamma$ -elimination of L-methionine and its derivatives and  $\alpha,\beta$ -elimination of L-cysteine and its derivatives [9]. With this approach, the following questions were addressed: (i) Can physiologically relevant concentrations of ASC inhibit cell growth *in vitro*? (ii) Are apoptosis or cell-cycle regulatory molecules affected under these conditions? (iii) Do lower levels of ASC affect DNA integrity?

## MATERIALS AND METHODS

### Chemicals

The following materials were purchased from commercial sources: Dulbecco's modified Eagle's medium and F-12 medium (Sigma, St. Louis, MO), adult bovine serum (Gemini Bioproducts, Calabasas, CA), insulin and epidermal growth factor (Intergen, Purchase, NY), gentamicin reagent solution (Gibco BRL, Grand Island, NY), agarose (GibcoBRL), Triton X-100 (Sigma), oligreen (Molecular Probes, Eugene, OR), glutaraldehyde (Sigma), crystal violet (Sigma), and MGL (Sigma; 1 unit releases 1.0  $\mu\text{mol}$  of  $\alpha$ -ketobutyrate per minute from L-methionine, pH 8.0, at 37°C). D,L-ASC was synthesized from D,L-selenocystine as described by Ip et al. [6]. Anti-p53, anti-Cip1/p21, anti-cyclin E, and anti-cyclin-dependent kinase (CDK) 2 antibodies; rabbit anti-mouse immunoglobulin- and goat anti-rabbit immunoglobulin-conjugated horseradish peroxidase secondary antibodies, and anti-mouse  $\beta$ -actin, which was used as a lane-loading control, were purchased from Santa Cruz Corp. (Indianapolis, IN). Anti-Kip1/p27, anti-cyclin D1, and anti-CDK4 antibodies were obtained from Neomarkers, Inc. (Fremont, CA). Anti-retinoblastoma tumor-suppressor protein (Rb) antibody was obtained from PharMingen/Transduction Laboratories (San Diego, CA). An enhanced chemiluminescence detection

system was purchased from Amersham Corp. (Arlington Heights, IL).

### Cell Culture

We elected to study the effects of ASC in a hyperplastic mammary epithelial cell line (TM12) because selenium has been reported to inhibit the progression of mammary hyperplasias *in vivo* [10]. This cell line has been shown to be responsive to selenium and has a wild-type *p53* gene, which we considered important, given our interest in factors affecting both cell-cycle progression and apoptosis. The mouse mammary hyperplastic epithelial cell line TM12 was obtained from the laboratory of Daniel Medina [11,12]. Cells were grown in Dulbecco's modified Eagle's medium and F-12 medium (1:1) containing 2% adult bovine serum, 10  $\mu\text{g}/\text{mL}$  insulin, 5  $\text{ng}/\text{mL}$  epidermal growth factor, and 5  $\mu\text{g}/\text{mL}$  gentamicin at 37°C in a humidified incubator containing 5%  $\text{CO}_2$ . The selenium content of complete medium was 60 nM, which is considered adequate for supporting the growth of cells in culture [13].

### Analysis of Cell Growth

The effect of ASC in the presence of MGL (ASC-MGL) on cell number was determined by evaluating the number of adherent cells as previously described [14]. Briefly, cells (1000 cells/well) were seeded in flat-bottomed 96-well plates in 100  $\mu\text{L}$  of culture medium. Twenty-four hours after initial seeding, cells were allowed to grow in either medium containing 0.1 U/mL MGL (no ASC) or the same medium supplemented with different concentrations of ASC. After 24 h of incubation, the medium was aspirated, and the cells were fixed for 15 min with 100  $\mu\text{L}$  of 1% glutaraldehyde in phosphate-buffered saline (PBS) per well. The fixative was removed and replaced with 150  $\mu\text{L}$  of PBS per well, and the plates were stored in PBS at 4°C. At the end of the experiment, all plates were stained simultaneously with 0.02% aqueous crystal violet solution (100  $\mu\text{L}/\text{well}$ ) for 30 min. The dye was removed by rinsing the plates with distilled water, and the residual water was then blotted out. The stain bound by the cells was redissolved in 70% ethanol (180  $\mu\text{L}/\text{well}$ ) while shaking the microplates for 2 h on a Titertek shaker (Titertek Instruments, Inc., Huntsville, AL). Absorbance was measured at 590 nm using a Thermo<sub>max</sub> Microplate Reader (Molecular Devices, Sunnyvale, CA).

### Comet Assay

The effect of ASC-MGL on DNA integrity was determined at 24 h of treatment by using the single-cell gel electrophoresis assay for assessing loss of DNA integrity, as described previously [15,16]. The assay, more commonly known as the comet assay, is a rapid and sensitive method for measuring DNA

strand breaks at the level of individual cells. Quantification of DNA integrity was performed by following the method of Collins et al. [16].

#### Apoptosis Counting

The effect of ASC-MGL on apoptosis was determined morphologically by fluorescent microscopy after labeling the cells with acridine orange and ethidium bromide, as described by Duke and Cohen [17]. Cells undergoing apoptosis in this system generally detach from the culture dish. Therefore, apoptosis was assessed by harvesting all cells, both floating and attached. Two hundred cells were counted for each apoptosis assay. In all experiments, each treatment was evaluated in duplicate. The effects of each treatment were studied in three independent experiments. The results of all three experiments were combined for the purpose of statistical analysis.

#### Expression of Cell-Cycle Regulatory Molecules by Western Blotting

In assessing the expression of cell-cycle regulatory molecules, only attached cells were studied. Cultures reaching a semiconfluent and logarithmically growing state were treated with 0.1 U/mL MGL  $\pm$  2.5  $\mu$ M ASC for 3, 6, 12, and 24 h. At the end of these periods, the medium was aspirated, the monolayer of cells was quickly washed two times with cold PBS, and then 0.3 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.2 U/mL aprotinin) per plate was added. After 15 min of incubation in lysis buffer on ice, the cells were scraped from the plate, and the mixture of buffer and cells was transferred to microfuge tubes and left in ice for an additional 15 min. The lysate was obtained by centrifugation for 15 min in a tabletop centrifuge at 4°C, and protein concentration in the clear supernatant was determined by the Bio-Rad detergent compatible protein assay (Bio-Rad, Hercules, CA). For western blotting of cell-cycle regulatory molecules, 40–100  $\mu$ g of protein lysate per sample was denatured with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (63 mM Tris HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue, and 5% 2-mercaptoethanol) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% or 12% gels, and the protein bands were blotted onto a membrane. The levels of p53, Cip1/p21, Kip1/p27, cyclin D1, CDK4, cyclin E, CDK2, and Rb were determined by using a specific primary antibody, followed by treatment with the appropriate peroxidase-conjugated secondary antibody, and visualized by using an enhanced chemiluminescence detection system. Signals were quantitated by scanning the film with ScanJet

(Hewlett Packard, Palo Alto, CA), and the intensity of the bands was analyzed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). To ensure that control cells (not treated with ASC) produced reproducible baseline protein levels, we carefully maintained cell cultures in a constant semiconfluent and logarithmically growing state throughout each experiment, as reported by others [18]. Multiple controls from each period (3–24 h) yielded similar protein signals (data not shown).

#### Statistical Analyses

Differences in cell growth, DNA integrity, and apoptosis in response to ASC-MGL treatment were evaluated by analysis of variance [19]. Post hoc comparisons across treatment conditions were made with the Bonferroni multiple-range test [19].

## RESULTS

#### Effects of ASC on Cell Growth, DNA Integrity, and Apoptosis

Table 1 shows the effect of increasing concentrations of ASC in the presence of 0.1 U/mL MGL on cell number, DNA integrity, and apoptosis at 24 h of exposure. Cell growth was significantly inhibited by ASC in a dose-dependent manner. The magnitude of the inhibition ranged from 14% at 0.5  $\mu$ M ASC to 49% at 2.5  $\mu$ M ASC. The comparison was made with control cells treated with MGL alone. When MGL was omitted from the medium, ASC (0.5–2.5  $\mu$ M) had no effect on cell growth; at 2.5  $\mu$ M ASC, cell growth was  $101 \pm 8\%$  of the appropriate control (no MGL, no ASC). We next determined the effect of ASC on DNA integrity as measured by the comet assay. As shown in Table 1, levels of ASC at 0.5–2.5  $\mu$ M in the presence of the MGL did not induce any change in DNA integrity. The response to ASC-MGL on the induction of apoptosis was also examined. Figure 1 shows that nucleoids from MGL-treated control cells were fully intact, whereas marked apoptotic bodies were observed in cells treated with ASC-MGL. ASC caused a marked dose-dependent increase in apoptosis at 24 h of exposure. The magnitude of the increase was statistically significant in comparison with MGL-treated control cells at each concentration of ASC ( $P < 0.01$ ). In the absence of MGL, the same concentrations of ASC had no effect on the rate of apoptosis ( $9.5 \pm 0.4$  at 2.5  $\mu$ M ASC).

#### Effects of ASC on Protein Levels of Cell-Cycle Regulatory Molecules

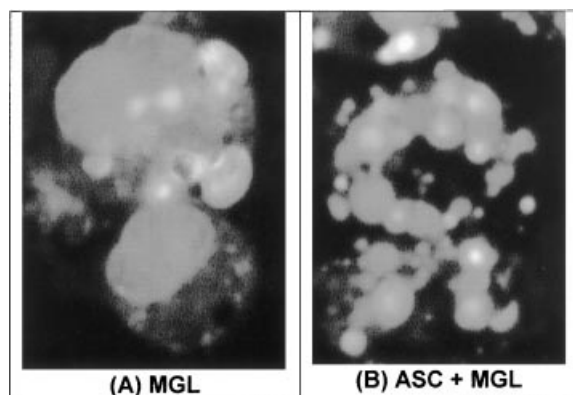
Previous work has shown that exposure to high levels of ASC, 50  $\mu$ M for 3–12 h, results in significant reductions in levels of phosphorylated Rb protein [20]. Therefore, we assessed the effects of much lower levels of ASC in the presence of MGL on levels

**Table 1. Effects of ASC-MGL on Cell Growth, DNA Integrity, and Apoptosis in TM12 Cells for 24 h\***

Selenium ( $\mu\text{M}$ )	Cell growth (%)	DNA integrity (AU)	Apoptosis (%)
0	100 <sup>a</sup>	51 $\pm$ 4.3 <sup>a</sup>	10 $\pm$ 0.3 <sup>a</sup>
0.5	86 $\pm$ 3.2 <sup>a</sup>	46 $\pm$ 3.9 <sup>a</sup>	14 $\pm$ 0.5 <sup>b</sup>
0.75	78 $\pm$ 4.6 <sup>b</sup>	44 $\pm$ 4.5 <sup>a</sup>	22 $\pm$ 1.0 <sup>c</sup>
1.0	59 $\pm$ 3.8 <sup>c</sup>	47 $\pm$ 4.6 <sup>a</sup>	25 $\pm$ 0.9 <sup>c</sup>
2.5	51 $\pm$ 3.7 <sup>c</sup>	59 $\pm$ 4.5 <sup>a</sup>	28 $\pm$ 0.9 <sup>d</sup>
Overall $P^{\dagger}$	< 0.001	> 0.1	< 0.01

\*Effect of treatment with ASC in the presence of 0.1 U/mL MGL on cell growth, DNA integrity, and apoptosis. For analyses of cell growth, eight replicates of each dose were analyzed in each of three experiments. The results of a representative experiment are presented. Data are expressed as percentage of untreated control, mean  $\pm$  SE ( $n = 8$ ). For analyses of DNA integrity determined by the comet assay, the data are expressed as arbitrary units (AU) of loss of DNA integrity, mean  $\pm$  SE ( $n = 9$ ). The score ranges from a minimum of zero to a maximum of 400. Two hundred cells were counted for each comet assay. In an experiment, each treatment was replicated three times. The effects of each treatment were studied in three independent experiments. The results of all three experiments were combined for the purpose of statistical analysis. For analyses of apoptosis, the data are expressed as a percentage of cells counted that were apoptotic, mean  $\pm$  SE ( $n = 6$ ). Two hundred cells were counted for each apoptosis assay. In an experiment, each treatment was evaluated in duplicate. The effects of each treatment were studied in three independent experiments. The results of all three experiments were combined for the purpose of statistical analysis.

<sup>†</sup>The overall  $P$  value refers to the results of the analysis of variance in which the effects of selenium treatment on cell growth, loss of DNA integrity, and apoptosis were compared with those observed in untreated cultures. When this  $P$  was smaller than 0.05, post hoc comparisons were performed. The results of these analyses are indicated by the superscripts. For each dose of the compound, values with different superscripts were statistically different at  $P < 0.05$ . No statistically significant difference was observed across different doses of the compound in the loss of DNA integrity.



**Figure 1.** Representative fluorescent photomicrographs showing the nucleoids of TM12 cells after 24 h of treatment with (A) 0.1 U/mL MGL alone or (B) 2.5  $\mu\text{M}$  ASC and 0.1 U/mL MGL. Nucleoids from MGL-treated control TM12 cells are fully intact, whereas marked apoptotic bodies are present in TM12 cells treated with 2.5  $\mu\text{M}$  ASC and 0.1 U/mL MGL.

of phosphorylated Rb. As shown in Figures 2 and 3, compared with MGL-treated control cells, ASC-MGL treatment suppressed the phosphorylation of Rb. A 40–70% reduction was found over the 24-h observation period.

Because Rb phosphorylation is initiated predominantly by cyclin D complexed to CDK4 in this cell line and is sustained by the cyclin E–CDK2 complex, the effect of ASC-MGL on total cellular levels of these individual proteins was assessed. A

chronic depression in cyclin D1 protein expression from 3 to 24 h of the exposure was observed, reaching a maximum of 80% reduction at 24 h. Levels of CDK4 were consistently lower in ASC-MGL–treated cells than in MGL-treated control cells over the same period of exposure. In general, levels of cyclin E were also lower in ASC-MGL–treated cells than in MGL-treated control cells; however, the magnitude of the reduction was much smaller than the effect observed on levels of cyclin D1. Levels of CDK2 were consistently lower in ASC-MGL–treated cells (Figures 2 and 3). Although the magnitude of the reduction was relatively small, it was comparable to the magnitude of change observed with CDK4.

To address further the question of why Rb phosphorylation was reduced by ASC, we next investigated the effects of ASC-MGL treatment on the expression of members of the Cip/Kip family of CDK inhibitors. ASC-MGL resulted in an increase in the amount of Kip1/p27 protein across 3–24 h (Figures 2 and 4). This result is consistent with the observation that levels of phosphorylated Rb were reduced during this same timeframe. Despite the fact that the ASC-MGL treatment had no effect on DNA integrity as measured by the comet assay (Table 1), we also assessed effects on p53 and on Cip1/p21, which is regulated by p53. As shown in Figures 2 and 4, compared with MGL-treated control cells, treatment with 2.5  $\mu\text{M}$  ASC in the presence of

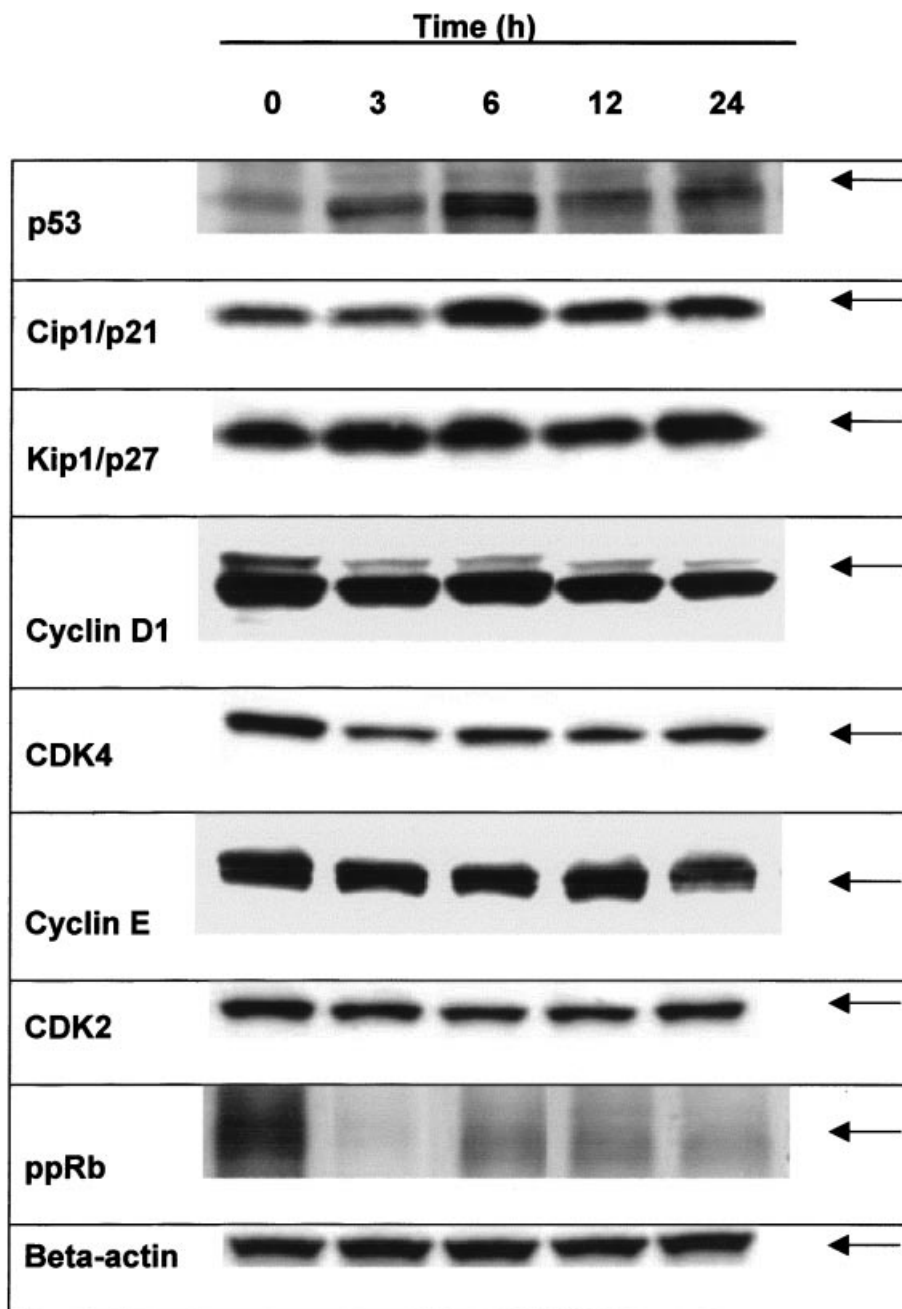


Figure 2. Effect on cell-cycle regulatory proteins p53, Cip1/p21, Kip1/p27, cyclin D1, CDK4, cyclin E, CDK2, and Rb in TM12 cells treated with 2.5  $\mu$ M selenium as ASC for 3–24 h in the presence of 0.1 U/mL MGL determined by western blot analyses. See Figures 3

and 4 for quantitative determinations of protein levels. Data are representative of three separate determinations. ppRb, hyperphosphorylated protein Rb.  $\beta$ -actin was used as an indicator for equality of lane loading.

0.1 U/mL MGL resulted in transient increases in the amount of p53 and Cip1/p21 protein at 6 h of treatment. Protein levels of p53 and Cip1/p21 were 2.8- and 2.7-fold higher, respectively, in ASC-MGL-treated cells than in MGL-treated control cells.

#### DISCUSSION

Our previous studies suggested that selenoamino acids are an excellent precursor for delivering and

generating a steady supply of selenium metabolites in vivo [1,2]. ASC is a stable selenoamino acid prodrug and requires the action of lyase enzymes to release the active selenium-containing species (presumably allylselenol) from the cysteine portion of the molecule [6]. Because lyase enzymes appear to be abundant in vivo in many tissues involved in the metabolism of selenium but are limiting in cultured mammary cells, an exogenous source of lyase

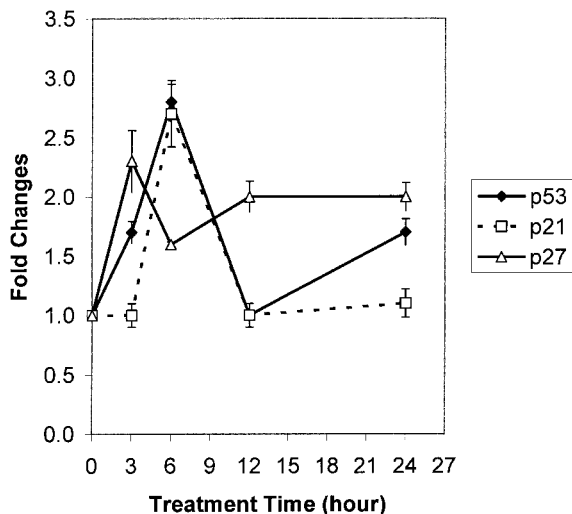


Figure 3. Relative changes in p53 and cyclin-dependent kinase inhibitory proteins shown in Figure 2 during treatment of TM12 cells with 2.5  $\mu$ M ASC in the presence of 0.1 U/mL MGL. Fold changes in protein were compared with those in MGL-treated control cells. Each point represents the mean  $\pm$  SE of triplicate cultures.

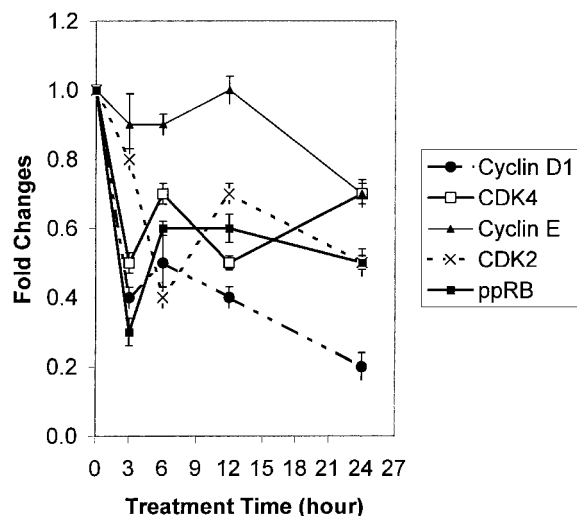


Figure 4. Relative changes in cyclins and CDK protein levels (Figure 2) during treatment of TM12 cells with 2.5  $\mu$ M ASC in the presence of 0.1 U/mL MGL. Fold changes in protein were compared with those of MGL-treated control cells. Each point represents the mean  $\pm$  SE of triplicate cultures.

activity, MGL, was used to overcome this problem, increasing the efficacy of ASC in vitro. The results presented in Table 1 provided a number of insights about the inhibition of cell growth by ASC under such a condition. The first significant observation is that in the presence of MGL, 2.5  $\mu$ M Se as ASC had the same effect as 50  $\mu$ M Se as ASC when the exogenous lyase was not provided. This finding implies that a metabolite of ASC released by MGL is exerting effects on these cells and that it is possible

to achieve in vitro effects of ASC at concentrations of Se in the medium similar to those achieved in vivo in plasma by a dietary concentration of ASC that inhibits mammary carcinogenesis [6]. This finding increases the likelihood that mechanisms identified in vitro, at lower concentrations of ASC, may also be operative in vivo at chemopreventive levels of the compound.

Given that physiologically relevant levels of ASC inhibited cell growth in vitro in the presence of MGL, we decided to reevaluate our previous observations about the effects of ASC on DNA integrity and its ability to induce apoptosis. As shown in Table 1, at a concentration of ASC that inhibited cell growth by approximately 50%, no loss of DNA integrity was observed in the attached cell population, whereas a modest loss of DNA integrity has been observed in response to the 50- $\mu$ M concentration of Se as ASC in this same cell line [7]. Despite this result, levels of apoptosis induced by 2.5  $\mu$ M ASC were similar to those induced by the higher concentration of Se as ASC [7]. Because we previously observed higher cellular levels of p53 in response to the 50- $\mu$ M concentration of ASC [20], cellular levels of p53 were also assessed to determine whether p53 levels were affected by ASC in the absence of DNA damage. Interestingly, we observed transient induction of p53 as well as Cip1/p21, with the increase in p53 protein preceding the increase in Cip1/p21. This finding implies that these two events may be related, a possibility that is supported by work done in other cell-culture model systems [21,22]. These events are also consistent with the observed increase in apoptosis. Thus, it appears that physiologically relevant levels of Se provided from ASC can increase cellular levels of p53 protein in the absence of DNA damage; however, whether or not the increased synthesis or stabilization of p53 protein can be shown to be directly involved in the increase of apoptosis observed after 24 h of exposure to ASC remains to be determined. In future work it will be instructive to clarify which caspase-mediated apoptosis induction pathway is induced by ASC and whether mitochondrial changes involving cytochrome C are involved. This information should assist in delineating the cell signaling pathway that is modulated by physiologically relevant concentrations of ASC.

Given previous reports that some Se compounds delay cells in the G<sub>1</sub> phase of the cell cycle [23–25], we hypothesized that treatment with ASC would inhibit Rb phosphorylation and affect cellular levels of regulatory proteins involved in its phosphorylation. The proteins in addition to Rb that were studied included cyclin D1 and CDK4, which are activated in early G<sub>1</sub>, initiating phosphorylation of Rb and progression toward S phase, and cyclin E and CDK2, which work to sustain Rb phosphorylation and entry into S phase [26,27]. As shown in Figures 2

and 3, phosphorylation of Rb was markedly lower in ASC-treated cells throughout the 24-h course of the experiment. Although our laboratory has recently reported that high levels of ASC reduce Rb phosphorylation [20], to our knowledge, no one has shown in vitro a reduction in Rb phosphorylation using a physiologically relevant concentration of selenium that does not influence DNA integrity. Consistent with this effect were the reductions observed in levels of cyclin D1 and, to a lesser extent, cyclin E [27]. These findings imply that lower levels of Rb were likely due to reduced kinase activity rather than to increased dephosphorylation of the protein. Consistent with this hypothesis, elevated cellular levels of Kip1/p27 would be expected to decrease the kinase activity of CDK2 and possibly CDK4, which in turn would result in an inhibition of Rb phosphorylation [28]. Overall, these data indicate that ASC treatment results in an anti-mitogenic intracellular environment and that this effect is unrelated to the induction by ASC of DNA damage detected by the comet assay.

In summary, the present data indicate that a metabolite of ASC, at physiologically relevant concentrations, can inhibit cell growth and induce apoptosis in the absence of an effect on DNA integrity. These effects are accompanied by significant reductions in phosphorylated levels of Rb. Candidate mechanisms by which these effects are achieved include the reduction in levels of G<sub>1</sub> cyclins and induction of CDK inhibitors such as Kip1/p27. The role of p53 and Cip1/p21 in mediating apoptotic cell death remains to be determined.

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