Biochemical Pharmacology, Vol. 60, pp. 1467–1473, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00461-5

In Vitro Effects of Se-Allylselenocysteine and Se-Propylselenocysteine on Cell Growth, DNA Integrity, and Apoptosis

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ABSTRACT. Two previously unevaluated selenium compounds, Se-allylselenocysteine (ASC) and Se-propylselenocysteine (PSC), have been shown recently to be active in the chemoprevention of experimentally induced mammary carcinogenesis. Other than their potential as chemopreventive agents, little is known about the pharmacological properties of these compounds. In this article, we report on the *in vitro* effects of ASC and PSC on cell growth inhibition, apoptosis, and the induction of DNA damage. The effects of ASC and PSC were examined in two mouse mammary epithelial cell lines derived from mammary hyperplasias. These cell lines, designated TM2H and TM12, have mutant or wild-type p53, respectively. It was observed that ASC but not PSC reduced, in a concentration- and time-dependent manner, the number of adherent cells in culture, and this suppressive effect was more prominent in TM12 than in TM2H cells. ASC was also found to induce alkaline-labile DNA damage and the oxidation of pyrimidines, and it also increased the rate of apoptosis. These changes were not seen by exposure to PSC or the sulfur analog of ASC. However, additional data obtained from the intact rat mammary gland suggest that the loss of DNA integrity induced by ASC might not be manifest *in vivo* at doses of ASC that inhibit carcinogenesis. BIOCHEM PHARMACOL **60**;10:1467–1473, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. selenium; selenocysteine; apoptosis; cell growth; DNA integrity

Our laboratories have been investigating the mechanisms that account for the cancer inhibitory activity of transient low molecular weight selenium metabolites, as reviewed in Ref. 1. Accumulating evidence from both *in vitro* and *in vivo* studies points to a monomethylated species of selenium generated during intermediary metabolism as being critically involved in mediating the growth inhibition of cancer cells [2]. Based on this observation, a stable, non-volatile pro-drug, MSC,‖ was developed that could deliver methylselenol to cells via a one-step β-lyase reaction, which also releases ammonia and pyruvate in the process [3]. This strategy has several advantages, which include circumventing some of the metabolic events that can lead to cell damage and delivering a form of selenium that does not accumulate significantly *in situ*. MSC has been shown to be active in inhibiting experimentally induced mammary carcinogenesis [4].

More recently, this line of investigation was extended to explore whether improved cancer inhibitory activity could be achieved by varying the aliphatic side chain in Se-alkylselenocysteine derivatives. A study comparing the effects of MSC, PSC, and ASC (structures shown in Fig. 1) in preventing mammary carcinogenesis demonstrated that MSC and PSC have similar efficacies, whereas the activity of ASC is significantly greater [5]. This observation is potentially very important to the field of selenium cancer chemoprevention if ASC exerts this inhibitory effect in the absence of toxicity.

It is well recognized that the biological activity of selenium is an expression of selenium in a variety of chemical forms, and not the element *per se* [6]. Over the last decade, we have used defined *in vitro* models to characterize the cellular responses to different chemical forms of selenium [7, 8]. *In vitro* models work well for this purpose because the specificity of the assay endpoints can be assessed without the complications of systemic metabolism. In this paper, we compare the effects of ASC and PSC on cell growth inhibition, DNA integrity, and apoptosis.

MATERIALS AND METHODS

Chemicals

The following materials were purchased from commercial sources: Dulbecco's modified Eagle's medium and F-12

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‖ *Abbreviations*: MSC, Se-methylselenocysteine; ASC, Se-allylselenocysteine; PSC, Se-propylselenocysteine; and SAC, *S*-allylcysteine.

Received 24 January 2000; accepted 5 May 2000.

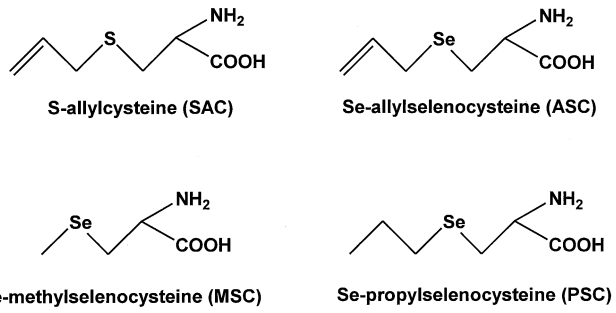

FIG. 1. Structures of SAC, ASC, MSC, and PSC.

medium (Sigma), adult bovine serum (Gemini Bioproducts), insulin and epidermal growth factor (Intergen), gentamicin reagent solution (GIBCO BRL), agarose (GIBCO BRL), Triton X-100 (Sigma), Oligreen (Molecular Probes), glutaraldehyde (Sigma), crystal violet (Sigma), α-keto-γ-methiol-butyric acid (Sigma), o-phenylenediamine (Sigma), and sodium selenite (Sigma). The D,L forms of ASC and PSC were synthesized from D,L-selenocystine as outlined in Ref. 5. D,L-SAC was provided by Eric Block (University of New York).

Cell Culture

The mouse mammary hyperplastic epithelial cell lines TM2H and TM12 were obtained from the laboratory of Daniel Medina [9]. Cells were grown at 37° in a humidified incubator containing 5% CO_2 in Dulbecco's modified Eagle's medium and F-12 medium (1:1 DMEM/F-12) containing 2% adult bovine serum, 10 μg/mL of insulin, 5 ng/mL of epidermal growth factor, and 5 μg/mL of gentamicin.

Determination of β-lyase Activity

We have reported this method previously in detail [5]. The only modification was the use of cytosol from cultured TM2H cells instead of tissue homogenate.

Analysis of Cell Growth Inhibition

The overall effect of ASC, PSC, and SAC on cell number homeostasis was determined by evaluating the number of adherent cells accumulating over different periods of time. This approach provides an estimate of the net effect of a compound on cell proliferation and cell death. The assay used crystal violet staining of adherent cells as an endpoint [10]. Briefly, cells (1000 cells/well) were seeded in flat-bottomed 96-well plates in 100 μL of culture medium. Twenty-four hours after initial seeding, cells were allowed to continue growing in either the same medium (no Se supplement) or in that medium supplemented with ASC or PSC at various concentrations of Se. The same approach was used in treating with SAC. After 24 hr of incubation, the medium was removed, and the cells were fixed for 15 min with 100 μL of 1% glutaraldehyde in PBS per well. The fixative was removed and replaced with 150 μL PBS/well, and the plates were stored under PBS at 4°. At the end of the experiment, all plates were stained simultaneously with 0.02% aqueous crystal violet solution (100 μL/well) for 30 min. Excess dye was removed by rinsing the plates with distilled water, and excess water was blotted out. The stain bound by the cells was redissolved in 70% ethanol (180 μL/well) while shaking the microplates for 2 hr on a Titertek shaker (Titertek Instruments, Inc.). Absorbance was measured at 590 nm using a $Thermo_{max}$ Microplate Reader (Molecular Devices).

Comet Assay

We adapted without modification the single-cell gel electrophoresis assay described previously by Singh *et al.* [11] as a method for assessing DNA damage. 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 damage was performed as recommended in Ref. 12. To produce standard curves of alkaline-labile DNA single-strand breaks, to which the effects of selenium compounds could be compared, TM2H cells were exposed to x-rays at various doses (0–500 rads) immediately prior to analysis.

A modified form of the comet assay was used for the specific detection of oxidative DNA base damage using a bacterial repair enzyme, endonuclease III, which recognizes oxidized pyrimidines and excises them, creating an alkaline-labile, apyrimidinic site [13]. To produce standard curves of DNA oxidation to which the effects of selenium compounds could be compared, TM2H cells were treated with methylene blue plus light before immersing in the lysis buffer. The conditions for exposure of the cells to methylene blue plus light were as described in Ref. 14.

In vivo *Assessment of DNA-Damaging Activity by Comet Analysis*

A 50-μm thick section of a formalin-fixed and paraffin-embedded mammary gland was placed in a test tube. The sample was deparaffinized in 10 mL xylene and hydrated gradually through a graded series of alcohols (100, 95, 70, and 50%, two changes for each). After washing and soaking for 10 min in two changes of distilled water, the deparaffinized section was transferred to a vial containing a pepsin solution [0.5% pepsin (Sigma 7012) in 0.9% saline with the pH adjusted to 1.5 using 2 M HCl]. Then, the vial was placed in a 37° water bath and incubated for 30 min with vortexing at 5-min intervals. After centrifuging at 800 *g* for 5 min at room temperature and removing the supernatant carefully with a Pasteur pipette, the tissue fragments were washed once with 10 mL PBS and resuspended in PBS for comet analysis as described above. Tissues from nine animals per treatment group were assessed.

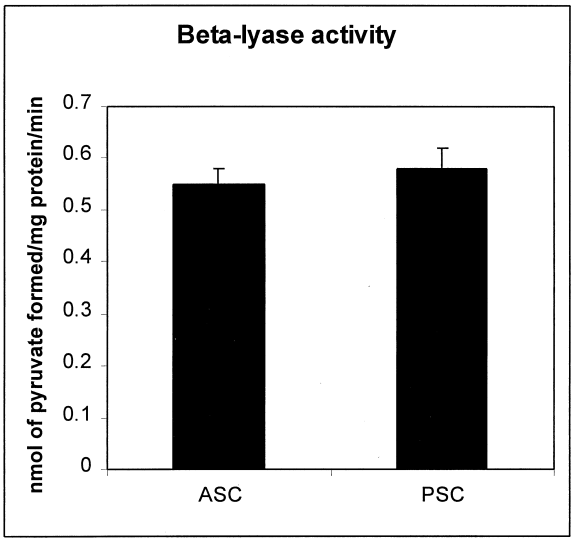

FIG. 2. Activities of β-lyase for ASC and PSC in TM mouse mammary epithelial cells. β-Lyase activity is expressed as nanomoles of pyruvate formed per milligram protein per minute. Data are means ± SEM, N = 3.

Apoptosis Counting

Effects of selenium compounds on apoptosis of cultured cells were determined morphologically by fluorescent microscopy after labeling with acridine orange and ethidium bromide as described by Duke and Cohen [15].

Statistical Analyses

Differences in the responses of the two cell lines to treatment with various concentrations and durations of treatment with selenium compounds were evaluated by factorial ANOVA [16]. *Post hoc* comparisons among treatment conditions were made using the Bonferroni multiple-range test [16].

RESULTS

β-lyase Activity

Prior to initiating the cell growth assays, we established that the TM series cell lines had β-lyase activity. The rates of pyruvate formation using ASC or PSC as substrates were similar: 0.55 ± 0.03 and 0.58 ± 0.04 nmol/min/mg protein, respectively (Fig. 2). Based on these β-lyase data, we were assured that our cell culture model had the enzymatic capability to generate selenols from ASC and PSC so that their biological activities could be evaluated.

Effects of ASC and PSC on Cell Growth

As shown in Table 1, cells were treated with increasing concentrations of selenium as ASC or PSC, and the effects on cell number were assessed at 24, 48, and 72 hr of exposure in two different cell lines, TM2H and TM12. We used both cell lines because TM12 has a wild-type p53, whereas TM2H has a non-functional mutant-type p53. We predicted comparable effects of these compounds on both cell lines unless the agent damaged DNA, in which case we hypothesized that a greater effect on cell growth inhibition would be evident in the wild-type p53 cell line. Several interesting observations emerged from the analysis of these data. In TM2H cells, cell growth was inhibited by ASC in a concentration-dependent manner at 48 and 72 hr of exposure, and the inhibitory effect of ASC was significantly greater at 72 hr relative to 48 hr of exposure ($P < 0.01$). At 24 hr of exposure, only an 11–18% inhibition of cell growth was observed across a wide range of ASC concentrations

TABLE 1. Effects of treatment with ASC or PSC on TM2H and TM12 cell growth inhibition*

	TM2H			TM12		
Selenium (μM)	**24 hr**	**48 hr**	**72 hr**	**24 hr**	**48 hr**	**72 hr**
ASC			% Untreated control			
25	89 ± 3.9[a]	92 ± 1.8[a]	58 ± 1.5[a]	84 ± 2.0[a]	37 ± 2.7[a]	13 ± 1.4[a]
50	89 ± 1.9[a]	60 ± 1.4[b]	31 ± 0.7[b]	85 ± 6.8[a]	20 ± 2.0[a,b]	5 ± 0.4[b]
100	84 ± 1.9[a]	44 ± 3.7[c]	16 ± 0.2[b]	60 ± 4.4[b]	11 ± 1.5[b]	3 ± 0.3[b]
200	82 ± 2.1[a]	29 ± 0.4[d]	8 ± 0.2[d]	46 ± 3.2[b]	7 ± 0.7[b]	2 ± 0.2[b]
Overall *P* value†	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
PSC						
25	104 ± 2.3	94 ± 2.8[a]	93 ± 0.6[a]	111 ± 6.3	97 ± 2.3	85 ± 1.9
50	107 ± 1.9	94 ± 2.6[a]	94 ± 1.1[a]	101 ± 6.1	94 ± 1.7	89 ± 1.2
100	99 ± 2.9	95 ± 1.5[a]	90 ± 1.4[a]	113 ± 6.3	96 ± 4.0	92 ± 3.1
200	98 ± 3.0	82 ± 2.2[b]	63 ± 0.8[b]	112 ± 7.9	105 ± 3.5	94 ± 2.6
Overall *P* value	NS	$P < 0.05$	$P < 0.05$	NS	NS	NS

* All experiments were repeated three times. In each experiment, eight replicates of each concentration of each compound were analyzed. The results of a representative experiment are presented. Data are expressed as percent of untreated control, means ± SEM (N = 8). Cell number was estimated as an absorbance value of crystal violet stained cells, λ = 590 nm. The absolute absorbance of TM2H cells in the untreated control was 0.079 ± 0.005, 0.165 ± 0.01, and 0.239 ± 0.01 at 24, 48 and 72 hr, respectively; the absolute absorbance of TM12 cells in the untreated control was 0.068 ± 0.004, 0.148 ± 0.01, and 0.259 ± 0.01 at 24, 48 and 72 hr, respectively.

† The overall *P* value refers to the results of the analysis of variance in which the effects of selenium treatment on cell growth inhibition were compared with cell growth inhibition observed in untreated cultures. When this *P* value was smaller than 0.05, *post hoc* comparisons were performed. The results of these analyses are indicated by the letter superscript. For each compound and treatment duration, values with different superscripts were statistically different, $P < 0.05$. NS not significant.

TABLE 2. DNA damage induced by selenium or sulfur compounds for 24 hr and x-ray in TM2H and TM12 cells*

Treatment	DNA damage (arbitrary units) TM2H	TM12
Control	78 ± 6[a]	59 ± 2[a]
ASC (μM)		
12.5	123 ± 5[b,c]	63 ± 4[a]
25	108 ± 4[c]	83 ± 6[a,b]
50	131 ± 8[b]	93 ± 3[b]
Overall *P* value†	$P < 0.01$	$P < 0.01$
PSC (μM)		
12.5	55 ± 3	58 ± 4
25	48 ± 2	57 ± 3
50	51 ± 2	58 ± 1
Overall *P* value	NS	NS
SAC (μM)		
12.5	46 ± 4	NS
25	49 ± 5	ND
50	47 ± 5	ND
Overall *P* value	NS	
X-ray (rads)		
50	144 ± 4	ND
100	190 ± 3	ND
250	275 ± 2	ND
500	344 ± 3	ND

* Data are expressed as arbitrary units of alkaline-labile DNA damage, means ± SEM (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. ND = not determined.

† The overall *P* value refers to the results of the analysis of variance in which the effects of selenium treatment on alkaline-labile DNA damage were compared with DNA damage observed in untreated cultures. When this *P* value was smaller than 0.05, *post hoc* comparisons were performed. The results of these analyses are indicated by the letter superscripts. For each compound and treatment duration, values with different superscripts were statistically different, $P < 0.05$. NS not significant.

(25 to 200 μM) (Table 1). The inhibitory effect of ASC on the accumulation of TM12 cells was of greater magnitude than that observed in TM2H cells (Table 1) ($P < 0.01$). The magnitude of this difference was 2- to 4-fold depending on the concentration and exposure times that were selected for comparison. Unlike the delay in response observed in TM2H cells, in TM12 cells the inhibitory effect of ASC was observed earlier, a concentration-dependent inhibition being evident at all three exposure durations ($P < 0.01$).

The effects of PSC on both cell lines were similar. In general, only a very modest inhibition of cell growth was noted, and the effect was observed only at the highest levels of exposure.

Effect of SAC on Cell Growth

To determine the specificity of ASC, the effect of the sulfur analog, SAC, was evaluated in TM2H cells and compared with that of ASC. SAC had no effect on cell growth inhibition (data not shown). This indicates that the substitution of selenium in place of sulfur has a dramatic effect on the activity of the compound in this assay system.

TABLE 3. Oxidative DNA damage induced by selenium or sulfur compounds for 24 hr and methylene blue in TM 2H cells

Treatment	Concn (μM)	DNA damage (arbitrary units)
Control		16 ± 6[a]
ASC	50	70 ± 4[b]
PSC	50	20 ± 8[a]
SAC	50	36 ± 6[c]
Methylene blue	1.0	34 ± 0.6
	2.5	61 ± 1.5
	5.0	90 ± 2.0
	7.5	122 ± 1.6
	10	138 ± 2.6

* Data are expressed as arbitrary units of DNA damage, means ± SEM (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. Values within a treatment condition with different letter superscripts are statistically different, $P < 0.05$.

Effect on DNA Integrity

In view of the differential effects of ASC on the growth of TM2H versus TM12 cells, we suspected that ASC might be inducing DNA damage at the levels used in the experiment. Given our previous work showing that some selenium compounds could induce DNA damage [7], we assessed both alkaline-labile DNA strand breaks and the presence of oxidized DNA bases using the comet assay. As shown in Table 2, ASC, but not PSC or SAC, induced alkaline-labile DNA damage in TM2H cells. The level of damage induced by 50 μM ASC was equivalent to that induced by exposure to approximately 50 rads of x-irradiation.

The effects of ASC and PSC on the induction of alkaline-labile damage also were examined in TM12 cells (Table 2). No change was detected following exposure to PSC compared with that seen in untreated cells. In ASC-treated cells, the increase in alkaline-labile damage was concentration dependent. However, the extent of damage was markedly lower in TM12 cells than in TM2H cells when both were exposed to the same concentration of ASC. Given the different p53 status of the two cell types, these data provided a rationale for the assessment of apoptosis, which is reported in a subsequent section.

We then proceeded to determine if ASC induced the oxidation of pyrimidines using a modification of the comet assay (Table 3). Exposure was limited to the 50 μM concentration of ASC, PSC, and SAC using the TM2H cell line, in which we had observed a greater sensitivity for alkaline-labile DNA damage. The effects of methylene blue plus irradiation (positive control) also were evaluated. Exposure of TM2H cells to 50 μM ASC or SAC induced oxidative damage ($P < 0.05$), whereas PSC was without effect. The level of DNA oxidation by ASC was similar to that induced by 2.5 μM methylene blue.

TABLE 4. Effect of treatment with selenium compounds for 24 hr on apoptosis*

Selenium (μM)	Apoptotic cells/100 cells counted	
	TM2H	TM12
ASC		
0	20 ± 0.3[a]	8.5 ± 0.7[a]
12.5	2.5 ± 0.3[a,b]	15.0 ± 0.9[b]
25	3.5 ± 0.5[b]	20.0 ± 0.8[c]
50	4.5 ± 0.4[c]	27.0 ± 0.8[d]
Overall *P* value†	$P < 0.01$	$P < 0.01$
PSC		
0	2.0 ± 0.3[a]	8.5 ± 0.7[a]
12.5	3.0 ± 0.4[a,b]	6.5 ± 0.8[a]
25	3.0 ± 0.3[a,b]	7.0 ± 0.8[a]
50	3.5 ± 0.5[b,c]	12.0 ± 0.8[b]
Overall *P* value	$P = 0.04$	$P < 0.01$
X-ray (rads)		
500, 3 hr	6.5 ± 0.4	30 ± 0.8
500, 6 hr	8.3 ± 0.5	30 ± 1.5

* Data are expressed as a percent of cells counted that were apoptotic, means ± SEM (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.

† The overall *P* value refers to the results of the analysis of variance in which the effects of selenium treatment on apoptosis were compared with the level of apoptosis observed in untreated cultures. When this *P* value was smaller than 0.05, *post hoc* comparisons were performed. The results of these analyses are indicated by the letter superscripts. For each compound and treatment duration, values with different superscripts were statistically different, $P < 0.05$. NS not significant.

Effect on Apoptosis

The effects of ASC and PSC on the induction of apoptosis at 24 hr of exposure were examined in both TM2H and TM12 cells (Table 4). In TM2H cells, the rate of apoptosis was low, about 2% in untreated cells. There was a statistically significant elevation of apoptosis by ASC and PSC, but the differences were small. In TM12 cells, PSC increased the rate of apoptosis only at the highest level of exposure (50 μM), whereas ASC induced a more marked concentration-dependent increase in apoptosis over the concentration range of 12.5 to 50 μM. The magnitude of the increase was statistically significant in comparison with untreated cells at each level of exposure. In TM12 cells, ASC was at least twice as effective as PSC in inducing apoptosis over the entire concentration range.

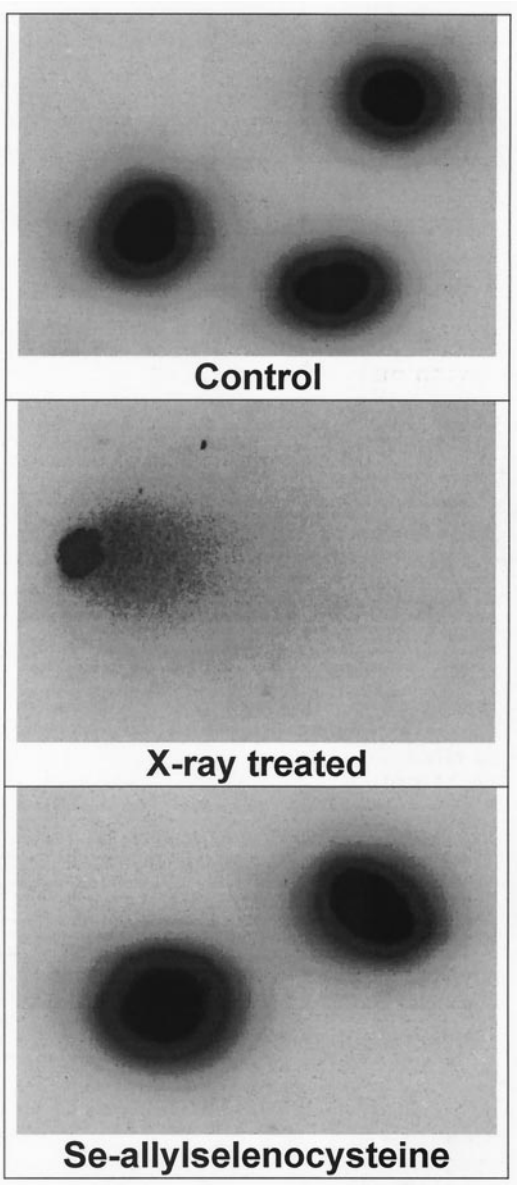

FIG. 3. Comet analysis on mammary tissue: Representative photomicrographs showing the nucleoids isolated from sections of formalin-fixed, paraffin-embedded mammary gland obtained from rats treated with 2 mg selenium as ASC/kg diet, or no supplemental selenium (control) or from rats irradiated immediately prior to euthanasia. Nucleoids from control or ASC-treated animals were fully intact, whereas extensive comet tailing was observed in mammary gland tissue from x-ray-treated rats.

In vivo *Assessment of DNA-Damaging Activity*

As an initial effort to determine whether the effects of ASC *in vitro* on DNA integrity are also manifest *in vivo,* the comet analysis was adapted for use on cells harvested from formalin-fixed, paraffin-embedded mammary tissue derived from a chemoprevention study, which showed that ASC has significant activity in suppressing methylnitrosourea-induced mammary carcinogenesis [5]. As a positive control to show that DNA damage can be detected in cells harvested from formalin-fixed, paraffin-embedded tissue, additional animals were subjected to x-irradiation (100 rads x-irradiation delivered at 80 rads/min with an orthovoltage instrument, General Electric Maximar 250; 225 kVp, 16 mA DC, 0.25 mm copper, 1 mm aluminum) *in vivo* according to our previously published method [17], and then the mammary tissue was processed for routine histology. In x-irradiated animals, greater than 90% of the cells showed comet tails (Fig. 3), whereas nucleoids isolated from control or ASC-treated rats were fully intact. While these data must be considered preliminary, they provide an initial indication that chemopreventive levels of ASC may not be

accompanied by DNA-damaging activity as was observed *in vitro*.

DISCUSSION

Our laboratories have reported recently that ASC has greater cancer chemopreventive activity than either PSC or MSC in a rat chemically-induced mammary tumor model [5]. However, the clinical implication of these potentially important results is unclear, since it is well recognized that for many selenium compounds, a narrow window separates the chemopreventive dose and the toxic dose [1, 6]. The studies reported in this paper were initiated to investigate the cytostatic and cytotoxic properties of ASC and PSC, about which little is known.

ASC behaved differently than PSC in inducing cell growth inhibition (Table 1), suggesting a unique activity(ies) of the Se-allyl moiety in comparison with Se-propyl. Moreover, the lack of effect of SAC implies specificity for selenium in achieving the inhibition of cell growth. The fact that ASC exerted differential effects on the two cell lines implied that ASC might be inducing cell damage. As can be seen from the data reported in Table 2, ASC induced alkaline-labile damage in TM2H cells. This effect was not observed when cells were treated with PSC or SAC, implying that this DNA-damaging activity is specific to ASC. Interestingly, levels of alkaline-labile DNA damage were much lower in TM12 cells treated with ASC than observed in TM2H cells. We conjectured that this effect might be accounted for by the induction of apoptosis in TM12 cells, which have a wild-type p53, and that damaged cells were being eliminated by apoptosis. This hypothesis was supported by data shown in Table 4. Rates of apoptosis were found to be significantly higher in TM12 cells exposed to ASC than those observed in comparably treated TM2H cells. The higher rate of apoptosis observed in the ASC-exposed TM12 cells could also explain the differential effect of ASC on cell growth inhibition reported in Table 1.

To better understand the implications of the DNA damaging activity observed in response to treatment with ASC, cells were exposed to a well characterized DNA-damaging agent, x-irradiation (Table 2). As expected, x-irradiation induced a dose-dependent increase in alkaline-labile damage. The steady-state level of damage induced by ASC was similar to the amount of damage induced in cells by 50 rads of radiation. This level of x-ray-induced damage generally is readily repaired, and is below the threshold associated with carcinogenic transformation when acutely administered [18, 19]. However, we cannot comment on its consequences if DNA damage is maintained at that level chronically.

We also assessed whether ASC induced DNA oxidation, since the oxidation of DNA bases is a recognized promutagenic lesion implicated in neoplastic transformation [20]. We adapted a recently published modification of the comet assay that detects the oxidation of pyrimidines for this purpose. Although the assay is not specific, it does serve as a dosimeter of overall oxidative events. For these experiments, we limited our analysis to a level of selenium provided as ASC that induced a significant amount of alkaline-labile damage. PSC did not induce an increase in oxidative DNA damage. Interestingly, a small increase in DNA oxidation was observed upon exposure to SAC, an effect that was magnified 2-fold at an equimolar concentration of ASC. The effect of ASC was below levels of oxidation by methylene blue plus irradiation that are associated with an increased rate of mutation [14]. These data imply that the DNA-oxidizing effect of ASC is not specific to selenium but does require the allyl moiety. An allyl-derived product that could be produced from both SAC and ASC is acrolein (propenal), formed as a result of Se- or S-dealkylation reactions. SAC undergoes sulfoxidation by microsomal flavin monooxygenase [21]. It is expected that the metabolism of ASC would result in the formation of the unstable selenoxide, which would undergo elimination of allylselenenic acid. Selenenic acids are known to undergo disproportionation to give a diselenide and a seleninic acid; unlike other diselenides and seleninic acids, the Se-allyl derivatives are not stable products. Allylseleninic acid is expected to eliminate SeO_2 and allyl alcohol (precursor of acrolein). Diallyl diselenide would extrude Se to form diallyl selenide, and after oxidation at the allylic carbon, the latter could undergo Se-dealkylation to form allylselenol and acrolein, as proposed for diallylsulfide [22]. The more facile breakdown of Se-allyl compounds compared with *S*-allyl analogs would likely cause relatively more acrolein formation from ASC than from SAC, in parallel with their effects on DNA integrity. This observation is consistent with the possibility that a common intermediate is formed during the cellular metabolism of both SAC and ASC, namely allyl alcohol. Oxidation of allyl alcohol would give the aldehyde derivative (propenal), known as acrolein. Acrolein has been reported to have DNA-damaging activity [23, 24].

Considering both the alkaline-labile DNA damage and the DNA oxidation data, we reasoned that at levels of ASC required *in vivo* for chemopreventive activity (2 mg/kg diet, which results in a plasma concentration of approximately 6 μM selenium [5]), levels of both active selenium metabolites and acrolein formed *in vivo* during metabolism would be unlikely to affect DNA integrity.

In a final set of experiments, we adapted the comet assay to fixed tissue specimens to assess whether chemopreventive levels of ASC induced detectable levels of DNA fragmentation *in vivo*. In support of our conjecture, we failed to observe comet tailing in tissues from animals that consumed ASC at chemopreventive doses. Additional experiments are planned to evaluate this issue further.

In summary, given the potent cancer inhibitory activity of ASC, it is critical that additional studies be done to evaluate the mechanisms that account for its DNA-damaging activity, and whether such effects are likely to be observed *in vivo*. Ongoing studies are directed toward

identifying the mechanisms by which ASC exerts its cancer chemopreventive activity.

This work was supported by Grant CA45164 from the National Cancer Institute.

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