[CANCER RESEARCH 60, 2882–2886, June 1, 2000]

In Vitro and *in Vivo* Studies of Methylseleninic Acid: Evidence That a Monomethylated Selenium Metabolite Is Critical for Cancer Chemoprevention[1]

Clement Ip,[2] Henry J. Thompson, Zongjian Zhu, and Howard E. Ganther

Department of Experimental Pathology, Roswell Park Cancer Institute, Buffalo, New York 14263 [C. I.]; Center for Nutrition in the Prevention of Disease, AMC Cancer Research Center, Denver, Colorado 80214 [H. J. T., Z. Z.]; and Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706 [H. E. G.]

ABSTRACT

Previous research suggested that the β-lyase-mediated production of a monomethylated selenium metabolite from Se-methylselenocysteine is a key step in cancer chemoprevention by this agent. In an attempt to affirm the concept, the present study was designed to evaluate the activity of methylseleninic acid, a compound that represents a simplified version of Se-methylselenocysteine without the amino acid moiety, thereby obviating the need for β-lyase action. The *in vitro* experiments showed that methylseleninic acid was more potent than Se-methylselenocysteine in inhibiting cell accumulation and inducing apoptosis in TM12 (wild-type p53) and TM2H (nonfunctional p53) mouse mammary hyperplastic epithelial cells, and these effects were not attributable to DNA damage, as determined by the comet assay. In general, methylseleninic acid produced a more robust response at one-tenth the concentration of Se-methylselenocysteine. It is possible that these cell lines may have only a modest ability to generate a monomethylated selenium species from Se-methylselenocysteine via the β-lyase enzyme. In contrast, methylseleninic acid already serves as a preformed active monomethylated metabolite, and this could be an underlying reason why methylseleninic acid acts more rapidly and exerts a more powerful effect than Se-methylselenocysteine *in vitro*. Interestingly, the distinction between these two compounds disappeared *in vivo*, where their cancer chemopreventive efficacies were found to be very similar to each other [in both methylnitrosourea and dimethylbenz(*a*)anthracene rat mammary tumor models]. The β-lyase enzyme is present in many tissues; thus, animals have an ample capacity to metabolize Se-methylselenocysteine systemically. Therefore, Se-methylselenocysteine would be expected to behave like methylseleninic acid if β-lyase is no longer a limiting factor. Taken together, the present *in vitro* and *in vivo* results provide strong evidence in support of our earlier hypothesis that a monomethylated selenium metabolite is important for cancer chemoprevention. Methylseleninic acid could be an excellent tool, especially for molecular mechanism studies in cell culture, and some of these attributes are discussed.

INTRODUCTION

Over the past decade, our collaborative group has been focusing part of our research effort on the identification of the active metabolite that is critical in selenium chemoprevention of cancer. It has been well documented that methylation is a prominent feature of selenite and selenomethionine metabolism (1). Both compounds are metabolized via different pathways to H_2Se, which is then sequentially methylated to methylselenol (CH_3SeH), dimethylselenide (CH_3-Se-CH_3), and trimethylselenonium ion [$(CH_3)_3Se^+$]. At high levels of selenium intake, dimethylselenide is expired in breath, whereas trimethylselenonium is excreted in urine (2). Toward the goal of understanding the significance of these methylated metabolites, our strategy was to select synthetic precursor compounds that were capable of delivering selenium to specific locations along the methylation pathway (3, 4). By this approach, we hoped to be able to pinpoint more closely the key metabolite that is involved in cancer protection. We found that any precursor that will directly generate a steady stream of methylselenol is more active than selenite or selenomethionine in tumor inhibition (5, 6). Thus, the facile endogenous production of monomethylated selenium is a critical factor in selenium chemoprevention. It should be noted that methylselenol is highly reactive and cannot be tested as is.

Se-methylselenocysteine, a lower homologue of selenomethionine, is one such compound that meets the criteria of a good precursor for generating methylselenol. Instead of entering the multistep transsulfuration pathway used by selenomethionine, Se-methylselenocysteine is acted upon immediately by the β-lyase enzyme to produce methylselenol (1, 7). We have used a chemically induced mammary tumor model in rats to evaluate the anticarcinogenic activity of Se-methylselenocysteine, selenite, and selenomethionine. All three compounds were added in the diet and administered to the animals under the same protocol. Our results indicated that there was a reproducible hierarchy of efficacy in the order of Se-methylselenocysteine > selenite > selenomethionine. The dose required for 50% tumor inhibition was 2 ppm Se for Se-methylselenocysteine, 3 ppm Se for selenite, and 4–5 ppm Se for selenomethionine (3, 6, 8–10).

As noted above, a β-lyase-mediated reaction is needed to free the monomethylated selenium species from Se-methylselenocysteine. To show conclusively the significance of this selenium metabolite in chemoprevention, we have investigated a novel compound, methylseleninic acid (CH_3SeO_2H). This is a simple but stable selenium-containing reagent with a single methyl group. It represents a stripped-down version of Se-methylselenocysteine without the amino acid moiety (hence, no need for β-lyase). In this paper, we report both the *in vitro* and *in vivo* effects of methylseleninic acid in comparison to that of Se-methylselenocysteine. The cell culture studies included experiments on cell accumulation, DNA integrity, and apoptosis. The animal studies were designed to evaluate the efficacy of mammary cancer chemoprevention, tissue selenium levels, and selenium bioavailability in maintaining selenoenzymes.

MATERIALS AND METHODS

Selenium Compounds. L-Se-Methylselenocysteine was obtained from Selenium Technologies, Inc. (Lubbock, TX). Methylseleninic acid was synthesized by the following method. Dimethyl diselenide (Aldrich Chemical Co.) was oxidized with 3% hydrogen peroxide at 65°C until the yellow color of the diselenide had disappeared (~3 mol of peroxide/mol of diselenide). The solution was adjusted to pH 7 with KOH and then applied to a column of Dowex 1 (chloride). After washing with water until a negative starch/iodide test was obtained, methylseleninic acid was eluted with 0.01 N HCl. The main starch/iodide-positive fractions were pooled, adjusted to pH 7 with KOH, and quantified by total selenium analysis and by iodometric titration of the iodine formed upon oxidation of iodide by methylseleninic acid. TLC on cellulose in butanol:acetic acid:water (5:2:3) showed a single starch/iodide-positive spot at R_f = 0.42. Reduction with excess borohydride gave a UV peak (252 nm; mM

Received 11/2/99; accepted 4/4/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Cancer Institute Grant CA 45164 and Roswell Park Cancer Institute Core Grant CA 16056.

[2] To whom requests for reprints should be addressed, at Department of Experimental Pathology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-8875; Fax: (716) 845-8100; E-mail: Clement.Ip@roswellpark.org.

extinction coefficient, 6.35) corresponding in wavelength and intensity to that of aliphatic selenolates.

Cell Culture. The two mouse mammary hyperplastic epithelial cell lines used in this study, TM2H and TM12, were obtained from the laboratory of Daniel Medina (11). Cells were grown at 37°C in a humidified incubator containing 5% CO_2 in DMEM and F-12 medium (1:1 ratio) containing 2% adult bovine serum (Gemini Bioproducts), 10 μg/ml insulin (Intergen), 5 ng/ml epidermal growth factor (Intergen), and 5 μg/ml gentamicin (Life Technologies, Inc.).

Analysis of Cell Number Accumulation. The assay, which provides an estimate of the net effect on cell proliferation and cell death, used crystal violet staining of adherent cells as an end point (12). Briefly, cells (1000 cells/well) were seeded in flat-bottomed 96-well plates in 100 μl of culture medium. Twenty-four h after initial seeding, cells were allowed to grow in either the control medium or the same medium supplemented with Se-methylselenocysteine or methylseleninic acid. After 24 h of treatment, the medium was shaken off, and the cells were fixed with 100 μl of 1% glutaraldehyde in PBS per well for 15 min. The fixative was then replaced by 150 μl of PBS, and the plates were kept at 4°C. All plates were stained simultaneously with 0.02% crystal violet solution (100 μl/well) for 30 min. The excess dye was removed by rinsing the plates with distilled water. The stain taken up by the cells was redissolved in 70% ethanol (180 μl/well); this was facilitated by shaking the microplates for 2 h on a Titertek shaker (Titertek Instruments, Inc., Huntsville, AL). Absorbance was measured at 590 nm using a $Thermo_{max}$ Microplate Reader (Molecular Devices, Sunnyvale, CA).

Comet Assay. We adapted the single-cell gel electrophoresis assay, described previously by Singh *et al.* (13), as a method for assessing DNA integrity. The technique, more commonly known as the comet assay, represents a rapid and sensitive way of measuring DNA strand breaks at the level of single cells. Cells were suspended in 80 μl of 1% low melting point agarose in PBS at 37°C and pipetted immediately onto a frosted glass microscope slide precoated with 1% high melting point agarose in PBS. The agarose was allowed to set in a thin layer on the microscope slide by protecting it with a coverslip at 4°C for 10 min. The slides were then immersed in a lysis solution (1% Triton X-100, 2.5 M NaCl, 0.1 M EDTA, and 10 mM Tris, pH 10.0) at 4°C for 1 h to remove proteinaceous materials while leaving "nucleoids," supercoiled DNA that is morphologically similar to that found in intact nuclei of cells. After lysis, the slides were subjected to an alkaline condition to promote unwinding of DNA at damaged sites by placing them in a 320-mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA at 4°C for 40 min before electrophoresis. During alkaline electrophoresis, DNA was attracted to the anode, but only segments of unwound DNA were free to migrate, extending from the nucleoid head to form the tail of a comet-like image. After washing the slides three times with neutralizing buffer (0.4 M Tris-HCl, pH 7.5), they were stained with Oligreen (Molecular Probes) to detect DNA single strand breaks. Assessments were made by the same observer using a fluorescence microscope (Axioskop 20; Carl Zeiss, Inc., Oberkochen, Germany) equipped with an excitation filter of 450–490 nm from a 150-W mercury lamp and a barrier filter of 510 nm. Samples were coded to avoid observer bias.

Quantification was achieved by visual scoring of 100 randomly selected comets per gel, two gels/slide, assigning them to one of five classes (0–IV), according to the degree of damage. Each single-cell DNA was given a value based on the prescribed class, and an overall score was derived for each gel, ranging from 0 (all of the single-cell DNA in class zero) to 400 (all of the single-cell DNA in class IV) arbitrary units. With practice, visual scoring is very quick and correlates well with computerized analysis (14).

Determination of Apoptosis. The induction of apoptosis in cultured cells was determined morphologically by fluorescent microscopy after labeling with acridine orange and ethidium bromide, as described by Duke and Cohen (15). Floating cells and enzymatically dissociated adherent cells were pooled and washed three times in PBS. The cells were centrifuged at 300 × *g*, and the pellet was gently resuspended in the culture media to make a suspension containing 1×10^6 cells/ml. A 25-μl-aliquot of this cell suspension was mixed with 1 μl of a dye solution containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide prepared in PBS. This mixture was placed on a slide, topped with a coverslip, and examined under a ×40 objective using a microscope equipped with epi-illumination and a fluorescein filter set (Axioskop 20; Carl Zeiss, Inc.). An observer blinded to the identity of the treatment sample scored at least 200 cells/sample. Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by acridine orange and ethidium bromide, respectively. The quantification of apoptotic cells was calculated as a percentage of the total number of cells counted.

Design of Mammary Cancer Prevention Experiment. Mammary cancer prevention by Se-methylselenocysteine or methylseleninic acid was studied with the use of both the MNU[3] and DMBA models. Pathogen-free female Sprague Dawley rats were purchased from Charles River Breeding Laboratories. They were fed the standard AIN-76A diet for 5 days to acclimatize them to the powder ration. The AIN-76 mineral mix provides 0.1 ppm Se (as sodium selenite) to the diet. Rats were given a single dose of 10 mg of MNU i.p. or 10 mg of DMBA intragastrically at 50 days of age. Each selenium compound was added to the basal diet at a final concentration of 2 ppm Se. The schedule of supplementation started at 3 days after carcinogen dosing and continued for 22 weeks until the end of the experiment. These diets were prepared weekly in the kitchen facility at the Roswell Park premise. Fresh food was provided to the animals every Monday, Wednesday, and Friday. Analysis of selenium recovered from the diets showed that both methylselenocysteine and methylseleninic acid remained stable in the diet at room temperature for at least 7–10 days. All animals were palpated weekly to determine the appearance, size, and location of mammary tumors. At necropsy, all tumors were excised and fixed for histological evaluation. Only confirmed adenocarcinomas are reported in the results. Tumor incidences at the final time point were compared by χ^2 analysis, and the total tumor yield was compared by frequency distribution analysis as described previously (16).

Tissue Selenium Analysis. Liver, kidney, mammary gland, and plasma samples ($n = 8$/group) were collected from the MNU carcinogenesis experiment at necropsy. They were immediately frozen in liquid nitrogen and stored at −80°C until ready for analysis. Selenium concentrations were determined by the fluorometric method of Olson *et al.* (17).

Repletion of Hepatic Selenoenzyme Activities. Hydrogen selenide (H_2Se) is generally accepted to be the precursor for the translational insertion of selenocysteine (coded by UGA) in the biosynthesis of selenoenzymes as exemplified by glutathione peroxidase and thioredoxin reductase (18). The ability of a given selenium compound to maintain selenoenzyme activity in an otherwise selenium-deficient animal is a measure of its bioavailability in replenishing the H_2Se pool (19). A selenium depletion/repletion protocol was used to assess the bioavailability of selenium from methylseleninic acid. Weanling rats were fed a selenium-deficient diet (20), which contained 0.01 ppm Se. After 4 weeks of feeding with this diet, they were given either sodium selenite (positive control) or methylseleninic acid for a duration of 3 weeks. Liver glutathione peroxidase and thioredoxin reductase activities were measured according to the methods as described by Paglia and Valentine (21) and Hill *et al.* (22), respectively.

Statistical Analyses. With the exception of the carcinogenesis results, all other data were evaluated by ANOVA with post hoc comparisons among treatment groups according to the method of Bonferroni (23).

RESULTS

***In Vitro* Studies.** The effects of Se-methylselenocysteine and methylseleninic acid on cell accumulation of TM2H and TM12 cultures are shown in Table 1. We used both cell lines because TM12 has a wild-type p53 whereas TM2H has a nonfunctional mutant-type p53. We predicted comparable effects of these compounds in both cell lines unless they caused DNA damage; in which case, we hypothesized that a greater effect would be evident in the wild-type p53 TM12 culture. Se-methylselenocysteine did not affect cell accumulation of TM2H cells and only marginally ($P > 0.05$) suppressed TM12 cells at the highest exposure level of 50 μM. In contrast, methylseleninic acid reduced cell number in both TM2H and TM12 cultures in a dose-dependent manner, and interestingly, these inhibitory effects were manifest in response to concentrations of methylseleninic acid that were ×10 lower than that of Se-methylselenocysteine. Because

[3] The abbreviations used are: MNU, methylnitrosourea; DMBA, dimethylbenz(*a*)anthracene.

Table 1 *Effects of different concentrations of Se-methylselenocysteine and methylseleninic acid on cell accumulation in TM2H and TM12 mammary cell cultures*

Treatment	TM2H cells[a]	TM12 cells[a]
Se-methylselenocysteine		
10 μM	99 ± 2.4[b]	98 ± 5.5[b]
25 μM	99 ± 2.1[b]	91 ± 9.3[b]
50 μM	97 ± 3.3[b]	84 ± 7.6[b]
Methylseleninic acid		
1 μM	83 ± 0.7[b]	77 ± 4.1[b]
2.5 μM	60 ± 1.0[c]	48 ± 3.9[c]
5 μM	44 ± 1.7[d]	46 ± 1.8[c]

[a] The data are expressed as a percentage of untreated control (mean ± SE; $n = 8$ replicate cultures). All of the results were obtained from 24-h cultures.
[b,c,d] Values that do not share common superscripts within an experimental condition are statistically different from each other ($P < 0.05$).

Table 2 *Effects of Se-methylselenocysteine and methylseleninic acid on DNA damage as measured by comet assay in TM2H and TM12 cells*[a]

Treatment	TM2H cells	TM12 cells
None	78 ± 6.0	59 ± 2.0
Se-methylselenocysteine		
25 μM	49 ± 4.0	56 ± 3.0
50 μM	51 ± 6.0	56 ± 3.0
None	79 ± 6.0	59 ± 1.7
Methylseleninic acid		
2.5 μM	55 ± 3.5	58 ± 1.3
5 μM	54 ± 4.1	62 ± 3.9

[a] The data are expressed as arbitrary units of alkaline-labile DNA damage, mean ± SE ($n = 9$). The score ranges from a minimum of zero to a maximum of 400. All of the results were obtained from 24-h cultures. The data were evaluated statistically to test the hypothesis that treatment with either Se-methylselenocysteine or methylseleninic acid would increase alkaline-labile DNA damage. No evidence was found to support this hypothesis.

the results were obtained from 24-h cultures, it is apparent that methylseleninic acid is able to produce a more rapid and powerful response compared with Se-methylselenocysteine.

We were concerned that the marked decrease in cell number by methylseleninic acid might reflect some form of genotoxicity, although the TM2H cells behaved similarly to the TM12 cells. To evaluate this possibility, we measured DNA integrity in single cells as determined by the comet assay (Table 2). As can be seen from the methodology section describing the quantification of the comet assay, the higher the score (maximum is 400), the greater is the extent of alkaline-labile DNA damage. Our data indicated that neither methylseleninic acid nor Se-methylselenocysteine produced any adverse effect on DNA integrity in TM2H or TM12 cells. To verify the sensitivity and reliability of the comet assay in detecting DNA damage, we had evidence showing that irradiation of cells by X-ray at doses of 250–500 rads produced scores in the range of 270–350 (data not shown).

Se-methylselenocysteine has been reported previously to induce a low level of apoptosis in the MOD mouse mammary tumor cell line (24). We were therefore interested in comparing the activity of Se-methylselenocysteine and methylseleninic acid in this respect. As shown in Table 3, the increase in apoptosis by Se-methylselenocysteine was small but statistically significant (about 2-fold or less) in both TM2H and TM12 cells. In contrast, methylseleninic acid produced a robust stimulation of a 6-fold increase in TM2H cells and a 3-fold increase in TM12 cells. Furthermore, these effects were achieved at concentrations of methylseleninic acid, which were an order of magnitude lower compared with Se-methylselenocysteine.

***In Vivo* Studies.** The efficacies of Se-methylselenocysteine and methylseleninic acid in mammary cancer prevention are summarized in Table 4. Note that in these animal experiments, the doses of the two compounds added to the diet were identical at a level of 2 ppm Se. Our results showed that in both the MNU and DMBA models of mammary carcinogenesis, the activities of Se-methylselenocysteine and methylseleninic acid in tumor suppression were comparable with each other; there were about 50–60% fewer tumors in the two selenium-treated groups than in the control group. The magnitude of tumor inhibition by Se-methylselenocysteine matched the results reported in our previous publications (6, 9, 10). Thus, we were confident that this study provided a reliable measure of the *in vivo* activity of methylseleninic acid.

Total tissue selenium analyses were performed in samples collected from the MNU carcinogenesis experiment, and the data are presented in Table 5. Treatment with Se-methylselenocysteine produced the expected elevation of selenium levels in liver, kidney, mammary gland, and plasma as reported previously (9). Supplementation with methylseleninic acid at the same dosage also produced a significant increase of selenium in tissues; however, the magnitude of the increase in general was consistently less than that found in the Se-methylselenocysteine group (refer to footnotes in table for statistical comparison). The reason for this difference will be discussed in a later section.

The ability of methylseleninic acid to replete the activity of selenoenzymes in selenium-deficient rats is presented in Table 6. The principle of the bioassay is based on the metabolism (at the whole animal level) of a given selenium compound to H_2Se, which is the precursor for selenocysteine incorporation into selenoproteins, such as glutathione peroxidase and thioredoxin reductase. As shown in Table 6, selenium deficiency caused a marked drop in the activities of both enzymes. Sodium selenite was used as a positive control because selenite selenium is highly bioavailable and will lead to full repletion of selenoenzymes at ~0.1 ppm. Methylseleninic acid also caused a dose-dependent repletion (from about 50 to 100% recovery) of selenoenzymes in the range of 0.05 to 0.2 ppm, suggesting that it is capable of being metabolized to H_2Se.

DISCUSSION

The most striking observation from these studies is the lack of congruency between the *in vitro* and *in vivo* results regarding the relative activities of Se-methylselenocysteine and methylseleninic acid. The cell culture data clearly demonstrated that methylseleninic acid was more potent than Se-methylselenocysteine in inhibiting growth and inducing apoptosis. These effects were not attributable to DNA damage. In general, methylseleninic acid produced a greater response at one-tenth the concentration of Se-methylselenocysteine. This difference disappeared *in vivo*, where the cancer chemopreventive efficacies of these two compounds were found to be comparable with each other. We will attempt to provide an explanation for these divergent effects.

As noted in the "Introduction," a β-lyase-mediated reaction is required to generate methylselenol from Se-methylselenocysteine. We

Table 3 *Effects of Se-methylselenocysteine and methylseleninic acid on apoptosis in TM2H and TM12 cells*

Treatment	TM2H cells[a] (%)	TM12 cells[a] (%)
None	2.0 ± 0.3[b]	8.5 ± 0.7[b]
Se-methylselenocysteine		
25 μM	3.0 ± 0.5[b,c]	9.0 ± 0.7[b]
50 μM	4.5 ± 0.6[c]	15.0 ± 1.3[c]
None	2.2 ± 0.5[b]	9.2 ± 0.4[b]
Methylseleninic acid		
2.5 μM	8.5 ± 0.5[c]	24.5 ± 0.9[c]
5 μM	12.8 ± 1.0[d]	33.5 ± 1.2[d]

[a] The data are expressed as a percentage of acridine orange- and ethidium bromide-stained apoptotic cells, mean ± SE ($n = 6$ replicates).
[b,c,d] The data were analyzed by factorial analysis of variance. The method of post hoc multiple comparisons was used to determine statistical significance among all treatment conditions (*i.e.*, dose and type of selenium compound) within a cell line. Values that do not share common superscripts are significantly different from each other ($P < 0.05$).

Table 4 *Mammary cancer prevention by Se-methylselenocysteine or methylseleninic acid in rats given a single dose of MNU or DMBA*

Carcinogen	Treatment[a]	Tumor incidence	Total no. of tumors	% inhibition[b]
MNU	None	29/30	94	
MNU	Se-methylselenocysteine	14/30[c]	40[c]	57%
MNU	Methylseleninic acid	16/30[c]	48[c]	52%
DMBA	None	26/30	85	
DMBA	Se-methylselenocysteine	13/30[c]	42[c]	51%
DMBA	Methylseleninic acid	13/30[c]	44[c]	48%

[a] Each selenium compound was supplemented in the diet at a concentration of 2 ppm Se for the entire duration of the experiment after carcinogen dosing.
[b] Percentage of inhibition of tumorigenesis calculated based on the total tumor number data.
[c] Significantly different compared with the corresponding control value ($P < 0.05$).

Table 5 *Tissue selenium levels in rats fed either Se-methylselenocysteine or methylseleninic acid*[a]

Treatment	Selenium concentration (μg/g or ml)[b]			
	Liver	Kidney	Mammary	Plasma
None	3.6 ± 0.17	5.5 ± 0.31	0.10 ± 0.01	0.46 ± 0.03
Se-methylselenocysteine	7.7 ± 0.53[c]	12.8 ± 0.84[c]	0.16 ± 0.01[c]	0.67 ± 0.04[c]
Methylseleninic acid	5.4 ± 0.41[c,d]	8.3 ± 0.70[c,d]	0.14 ± 0.01[c]	0.47 ± 0.04[d]

[a] The animals were from the MNU mammary carcinogenesis experiment described in Table 4.
[b] Results are expressed as mean ± SE ($n = 8$).
[c] $P < 0.05$, compared with the corresponding control value.
[d] $P < 0.05$, compared with the corresponding value from the Se-methylselenocysteine group.

have evidence that both TM2H and TM12 cells express this enzyme activity (25). However, this activity is measured in a test tube under optimal conditions using a subcellular fraction as the enzyme source. Without actually quantifying the production of methylselenol when cultured cells are exposed to different concentrations of Se-methylselenocysteine, it is not possible to assess the physiological rate of this enzymatic reaction. Methylseleninic acid, on the other hand, delivers a monomethylated selenium species directly to cells. This may be an underlying reason why methylseleninic acid acts more rapidly and exerts a more powerful effect than Se-methylselenocysteine *in vitro*.

The β-lyase enzyme is present in intestine, liver, kidney, and mammary gland (9); there could be other tissues that also express this enzyme. Thus, methylselenol is capable of being generated at different organ sites. It is likely that animals have ample capacity to release the CH_3Se moiety from Se-methylselenocysteine. Thus, the chemical distinction between Se-methylselenocysteine and methylseleninic acid may no longer be crucial when either agent is given as a dietary supplement for cancer chemoprevention. There are alternative, competing reactions that Se-methylselenocysteine could undergo, such as *N*-acetylation, transamination, and other reactions involving either the amino acid structure or the selenium atom. In theory, the formation of some other amino acid or peptide derivative from Se-methylselenocysteine might be required for chemopreventive activity. The current evidence showing that chemopreventive activity is obtained independent of the amino acid structure provides firm support for our earlier hypothesis that a simple monomethylated selenium metabolite is a proximal metabolite for cancer prevention.

Once taken up by cells, methylseleninic acid is likely to undergo facile reduction to methylselenol via nonenzymatic and enzymatic processes. Nonenzymatic reaction with three molecules of thiol (RSH) would form equimolar amounts of selenenylsulfide (CH_3-Se-SR) and disulfide products, based on the studies of Kice and Lee (26): $CH_3SeO_2H \rightarrow CH_3Se(O)$-SR $\rightarrow CH_3SeOH \rightarrow CH_3Se$-SR. Further reduction of the methylselenenylsulfide to methylselenol would occur with excess thiol. Under cellular conditions where GSH is the major thiol, a CH_3Se-SG selenenylsulfide intermediate would be formed that is expected to undergo NADPH-linked reduction by glutathione reductase to methylselenol, similar to the reduction of GS-Se-SG to hydrogen selenide (1). It should also be noted that the above reactions may not be the only pathway. Chemically, heat-induced disproportionation of methylseleninic acid to release formaldehyde, methylselenol, and inorganic selenium has been described (27): $2CH_3SeO_2H \rightarrow CH_2O + CH_3SeH + SeO_2 + H_2O$. Incidentally, the formation of CH_3SeH from methylseleninic acid may also be inferred from the selenium bioavailability study of Table 6. The ability of methylseleninic acid to replete selenoenzymes implies that a small amount of H_2Se is produced, and one source of H_2Se is from the demethylation of CH_3SeH (3).

As shown in Table 5, total tissue selenium levels were consistently lower in animals given methylseleninic acid compared with those given Se-methylselenocysteine. This is probably attributable to the rapid systemic clearance of methylseleninic acid through its facile conversion to methylselenol, which is then further metabolized to dimethylselenide and trimethylselenonium for excretion in breath and urine, respectively. In contrast, the generation of methylselenol from Se-methylselenocysteine is mediated via the slower β-lyase reaction; this enzyme has a fairly high K_m value (9). Although Se-methylselenocysteine and methylseleninic acid are equally effective in cancer chemoprevention, the former may be more desirable to use in human trials because it is expected to generate a steady stream of methylselenol *in situ* and thus avoids the garlic breath of exuberant dimethylselenide elimination as discussed above.

What have we learned from the experiments with methylseleninic acid? This reagent could be an excellent tool for molecular mechanism studies in cell culture: (*a*) unlike selenite, methylseleninic acid does not produce a copious amount of H_2Se, which is associated with a variety of genotoxic effects in cells (28, 29); (*b*) the use of methylseleninic acid circumvents the need for a β-lyase reaction, as is the case with Se-methylselenocysteine. This allows methylseleninic acid to be evaluated in cells in which β-lyase is a limiting factor; (*c*) methylseleninic acid elicits a greater response than Se-methylselenocysteine, thus increasing the sensitivity of the model system. As shown in the present study, the mammary hyperplastic epithelial cells are only minimally affected by Se-methylselenocysteine. In contrast, the effective concentration of methylseleninic acid needed in the culture medium is much lower and is similar to that seen under physiological conditions; (*d*) because methylseleninic acid is already a preformed monomethylated selenium, it could be used to study the modulation of target proteins with cysteine clusters along a redox mechanism, as proposed by Ganther (30) in a recent review. These attributes of methylseleninic acid will be examined in depth in our future research.

Table 6 *Ability of methylseleninic acid to replete glutathione peroxidase and thioredoxin reductase activities in liver of selenium-deficient rats*[a]

Group	Glutathione peroxidase (%)	Thioredoxin reductase (%)
Selenium-adequate control rats	100	100
Selenium-deficient rats	1.5 ± 0.1	5.3 ± 0.3
Repletion with		
Sodium selenite at 0.1 ppm Se (positive control)	97.5 ± 5.8	94.7 ± 6.6
Methylseleninic acid at		
0.05 ppm Se	56.1 ± 4.2	51.3 ± 3.9
0.1 ppm Se	77.8 ± 6.3	72.7 ± 6.1
0.2 ppm Se	106.2 ± 6.8	98.4 ± 7.5

[a] The data are expressed as percentages of control activity in the liver of rats fed a selenium-adequate diet (set as 100%). $n = 8$ rats/group.

ACKNOWLEDGMENTS

We are grateful to Cathy Russin, Tamora Loftus, and Weiqin Jiang for technical assistance.

REFERENCES

1. Ganther, H. E., and Lawrence, J. R. Chemical transformations of selenium in living organisms. Improved forms of selenium for cancer prevention. Tetrahedron, *53:* 12299–12310, 1997.
2. Vadhanavikit, S., Ip, C., and Ganther, H. E. Metabolites of sodium selenite and methylated selenium compounds administered at cancer chemoprevention levels in the rat. Xenobiotica, *23:* 731–745, 1993.
3. Ip, C., and Ganther, H. E. Relationship between the chemical form of selenium and anticarcinogenic activity. *In:* L. Wattenberg, M. Lipkin, C. W. Boone, and G. J. Kelloff (eds.), Cancer Chemoprevention, pp. 479–488. Boca Raton, FL: CRC Press, 1992.
4. Ip, C. Lessons from basic research in selenium and cancer prevention. J. Nutr., *128:* 1845–1854, 1998.
5. Ip, C., and Ganther, H. E. Activity of methylated forms of selenium in cancer prevention. Cancer Res., *50:* 1206–1211, 1990.
6. Ip, C., Hayes, C., Budnick, R. M., and Ganther, H. E. Chemical form of selenium, critical metabolites, and cancer prevention. Cancer Res., *51:* 595–600, 1991.
7. Andreadou, I., Menge, W. M. P. B., Commandeur, J. N. M., Worthington, E. A., and Vermeulen, N. P. E. Synthesis of novel Se-substituted selenocysteine derivatives as potential kidney selective prodrugs of biologically active selenol compounds: evaluation of kinetics of β-elimination reactions in rat renal cytosol. J. Med. Chem., *39:* 2040–2046, 1996.
8. Ip, C., and Hayes, C. Tissue selenium levels in selenium-supplemented rats and their relevance in mammary cancer protection. Carcinogenesis (Lond.), *10:* 921–925, 1989.
9. Ip, C., Zhu, Z., Thompson, H. J., Lisk, D., and Ganther, H. E. Chemoprevention of mammary cancer with Se-allylselenocysteine and other selenoamino acids in the rat. Anticancer Res., *19:* 2875–2880, 1999.
10. Ip, C., and Ganther, H. E. Comparison of selenium and sulfur analogs in cancer prevention. Carcinogenesis (Lond.), *13:* 1167–1170, 1992.
11. Medina, D., Kittrell, F. S., Liu, Y. J., and Schwartz, M. Morphological and functional properties of TM preneoplastic mammary outgrowths. Cancer Res., *53:* 663–667, 1993.
12. Bernhardt, G., Reile, H., Birnbock, H., Sprub, T., and Schonenberger, H. Standardized kinetic microassay to quantify differential chemosensitivity on the basis of proliferative activity. J. Cancer Res. Clin. Oncol., *118:* 35–43, 1992.
13. Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res., *175:* 184–191, 1988.
14. Collins, A. R., Duthie, S. J., and Dobson, V. L. Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis (Lond.), *14:* 1733–1735, 1993.
15. Duke, R. C., and Cohen, J. J. Morphological and biochemical assays of apoptosis. *In:* J. E. Coligan and A. M. Kruisbeak (eds.), Current Protocols in Immunology, pp. 3.17.1–3.17.16. New York: John Wiley and Sons, 1992.
16. Horvath, P. M., and Ip, C. Synergistic effect of vitamin E and selenium in chemoprevention of mammary carcinogenesis in rats. Cancer Res., *43:* 5335–5341, 1983.
17. Olson, O. E., Palmer, I. S., and Carey, E. E. Modification of the official fluorometric method for selenium in plants. J. Assoc. Off. Anal. Chem., *58:* 117–121, 1975.
18. Stadtman, T. C. Selenocysteine. Annu. Rev. Biochem., *65:* 83–100, 1996.
19. Burk, R. F., and Hill, K. E. Regulation of selenoproteins. Annu. Rev. Nutr., *13:* 65–81, 1993.
20. Ip, C., and Lisk, D. J. Bioavailability of selenium from selenium-enriched garlic. Nutr. Cancer, *20:* 129–137, 1993.
21. Paglia, D. E., and Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med., *70:* 158–169, 1967.
22. Hill, K. E., McCollum, G. W., Boeglin, M. E., and Burk, R. F. Thioredoxin reductase activity is decreased by selenium deficiency. Biochem. Biophys. Res. Commun., *234:* 293–295, 1997.
23. Snedecor, G. J. W., and Cochran, W. G. Statistical Methods, Ed. 6, pp. 135–317. Ames, Iowa: Iowa State University, 1967.
24. Lu, J., Pei, H., Ip, C., Lisk, D. J., Ganther, H., and Thompson, H. J. Effect of an aqueous extract of selenium-enriched garlic on *in vitro* markers and *in vivo* efficacy in cancer prevention. Carcinogenesis (Lond.), *17:* 1903–1907, 1996.
25. Zhu, Z., Jiang, W., Ganther, H. E., Ip, C., and Thompson, H. J. *In vitro* effects of Se-allylselenocysteine and Se-propylselenocysteine on cell growth, DNA integrity and apoptosis. Biochem. Pharmacol., in press, 2000.
26. Kice, J. L., and Lee, T. W. S. Oxidation-reduction reactions of organoselenium compounds. 1. Mechanism of the reaction between seleninic acids and thiols. J. Am. Chem. Soc., *100:* 5094–5102, 1978.
27. Bird, M. L., and Challenger, F. Potassium alkaneselenonates and other alkyl derivatives of selenium. J. Chem. Soc., *1942*: 570–574, 1942.
28. Lu, J., Jiang, C., Kaeck, M., Ganther, H., Vadhanavikit, S., Ip, C., and Thompson, H. Dissociation of the genotoxic and growth inhibitory effects of selenium. Biochem. Pharmacol., *50:* 213–219, 1995.
29. Kaeck, M., Lu, J., Strange, R., Ip, C., Ganther, H. E., and Thompson, H. J. Differential induction of growth arrest inducible genes by selenium compounds. Biochem. Pharmacol., *53:* 921–926, 1997.
30. Ganther, H. E. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. Carcinogenesis (Lond.), *20:* 1657–1666, 1999.

In Vitro and *in Vivo* Studies of Methylseleninic Acid: Evidence That a Monomethylated Selenium Metabolite Is Critical for Cancer Chemoprevention

Clement Ip, Henry J. Thompson, Zongjian Zhu, et al.

Cancer Res 2000;60:2882-2886.