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# Methylseleninic acid, a potent growth inhibitor of synchronized mouse mammary epithelial tumor cells *in vitro*

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**Abstract**

Selenium compounds have been shown to be effective chemopreventive agents in several animal models and in cultured cells *in vitro.* It has been proposed that compounds able to generate monomethyl Se have an increased potential to inhibit cell growth. To test this hypothesis, methylseleninic acid (MSeA) and other compounds that could generate methylselenol rapidly were compared with Se compounds that do not generate monomethyl Se, using a well-characterized synchronized TM6 mouse mammary epithelial tumor model *in vitro.* MSeA at a low micromolar concentration inhibited TM6 growth after 10- to 15-min treatment times. Cells resumed growth after 24 hr but remained sensitive to the fresh addition of monomethyl Se-generators. Dimethyl selenide (DMSe), a putative metabolite of methylselenol, was inactive. Cells treated with 5 $\mu$M MSeA were arrested in $G_{1.}$ The effects of 5 $\mu$M MSeA on gene expression were evaluated using the Atlas mouse cDNA expression array. A 10-min exposure with MSeA caused a 2- to 3-fold change in the expression of three genes: laminin receptor 1 (decreased), integrin beta (decreased), and *Egr-1* (increased). The results provide experimental support for the hypothesis that monomethylated forms of Se are the critical effector molecules in Se-mediated growth inhibition *in vitro.* 

*Keywords:* Methylseleninic acid; Mouse mammary tumor; Growth inhibition

## 1. Introduction

Selenium compounds have been shown to inhibit tumorigenesis in experimental animals [1–5], and recent studies indicate that supplemental Se reduces cancer risk in humans [6]. Several organic Se compounds have been recognized as effective chemopreventive agents against the development of tumors in the mammary gland, lung, colon, and prostate [2,7–13]. The chemopreventive ability of Se as an antitumor agent depends on its chemical form [14], and metabolism of the parent compound is critical to provide the reactive form. Monomethylated forms of Se have significant effects on carcinogenesis [15]. MSC is a good precursor for generating monomethylated Se as it can be converted directly to methylselenol [$CH_3SeH$] via a cysteine conjugate $\beta$ -lyase reaction [16]. MSC may also be oxidized to MSC selenoxide, which in the presence of cysteine conjugate $\beta$ -lyase can give rise to methylselenenic acid (unstable) leading to methylselenenylsulfide and finally to methylselenol (Fig. 1). MSC is reported to be a better chemopreventive agent than either selenomethionine or selenite at 1–3 ppm [14,15]. In contrast, DMSeO undergoes rapid reduction to DMSe, and it has a low chemopreventive activity [17] since DMSe is rapidly expired in breath [18].

A monomethylated form of Se that is not too volatile is needed for *in vitro* studies that examine mechanisms of Se anti-cancer action. Therefore, MSeA [$CH_3SeO_2H$], an oxidized monomethylated form of Se, may be suitable for use directly or as a precursor of methylselenol (Fig. 1). The free acid ionizes above pH 5 to the anion, methylseleninate [$CH_3SeO_2^-$], which is practically odorless. A stock solution of methylseleninate at pH 7, therefore, is convenient for use and is quite stable if protected from microbiological action or reducing agents. It is a fairly strong oxidizing agent, like sodium selenite, and will readily undergo reduction, giving

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*Abbreviations:* DMEM:F12, Dulbecco's Modified Eagle's Medium: Nutrient mixture F12; DMSe, dimethyl selenide; DMSeO, dimethyl selenoxide; DMSeS, dimethyl selenenylsulfide; DMSeSe, dimethyl diselenide; DMSS, dimethyl disulfide; MSC, Se-methylselenocysteine; MSeA, methylseleninic acid; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide.

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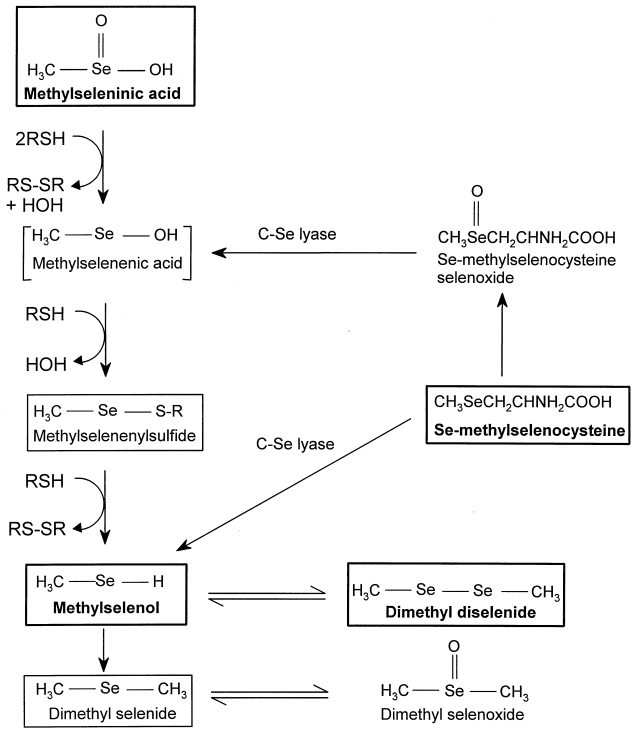


Fig. 1. Schematic presentation for the formation of various methylselenides. MSeA forms methylselenol through a series of reactions with methylselenenylsulfide as an intermediate compound. MSC, on the other hand, can be converted to methylselenol by the cysteine conjugate $\beta$-lyase. DMSe can be reduced to methylselenol, while DMSeO does not form methylselenol.

rise to methylselenol. Methylselenol then undergoes oxidation or methylation to give DMSeSe and DMSe, respectively. According to the hypothesis of Ip and Ganther [14, 15,19], simple monomethylated forms of Se should have strong growth inhibitory activity at low concentrations and exert effects rapidly. We sought to test this hypothesis by examining the effects of MSeA in a well-defined *in vitro* system, using TM6 mammary epithelial cells.

## 2. Materials and methods

### *2.1. Synthesis of Se compounds*

DMSeO was synthesized as described earlier [17]. MSeA was prepared by combining DMSeSe (Aldrich Chemical Co.) in methanol with hydrogen peroxide (3%) at 65° until the yellow color of the diselenide had disappeared. 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, MSeA was eluted with 0.01 N HCl. The main starch/iodide-positive fractions were pooled, adjusted to pH 7 with KOH, and analyzed. TLC on cellulose in butanol:acetic acid:water (5:2:3) showed a single starch/iodide positive spot of $R_f$ = 0.42. Reduction with excess borohydride gave a UV peak (252 nm, millimolar extinction coefficient 6.35) corresponding in wavelength and intensity to that of aliphatic selenolates. DMSeS was synthesized by reacting methylselenenyl bromide (prepared by reacting DMSeSe with bromine) with methane thiol [20]. Analysis of the reaction mixture by $C_{18}$ reversed phase HPLC (75% methanol) using a diode array detector showed successive peaks having UV spectra corresponding to S-S, Se-S, and Se-Se derivatives [20]. DMSeS was obtained in pure form by separating it from the symmetrical products using preparative HPLC in 75% methanol. The isolated product was stable when stored at −20°.

### *2.2. Cell culture and synchronization*

The TM6 tumor cell line was derived from the COMMA-D mouse mammary epithelial cell line [21]. TM6 cells were maintained in DMEM:F12 (1:1) medium supplemented with 5 $\mu$g/mL of insulin, 5 ng/mL of epidermal growth factor, 2% adult bovine serum, and 5 $\mu$g/mL of gentamicin at 37° in a humidified atmosphere in the presence of 5% $CO_2$. For experiments, cells were seeded at $6.6 \times 10^3/cm^2$ and were synchronized as described earlier [9]. Cells were released from arrest by feeding them complete medium (containing growth factors and serum) for 6 hr at which time these cells were treated separately with each of the five selenium compounds: MSeA, DMSeSe, DMSeS, DMSe, and DMSeO (all at a 5 $\mu$M concentration for [Se] for all the experiments). Untreated cells were taken as control cells and were analyzed in the following assays at various times after Se exposure.

### *2.3. Cell cycle analysis*

Synchronized TM6 cells were treated with MSeA (5 $\mu$M) for various time intervals followed by enzymatic dissociation and fixation in 95% alcohol overnight. The untreated control and MSeA-treated cells were stained with propidium iodide (50 $\mu$g/mL), incubated with RNase (100 $\mu$g/mL) at 37° for 30 min, and examined by flow cytometry.

### *2.4. [$^3$H]Thymidine assay*

[$^3$H]Thymidine incorporation into DNA was used to measure cell proliferation activity. Synchronized TM6 cells were treated with MSeA, DMSeSe, DMSeS, DMSe, and DMSeO (all at 5 $\mu$M), rinsed with DMEM:F12 medium, and pulsed with [*methyl*-$^3$H]thymidine (1 $\mu$Ci/well) for 1 hr following varied time intervals for treatment, as described in Results. Untreated TM6 cells were taken as controls. Radioactivity incorporated into acid-precipitable material was counted as described previously [22]. The data are depicted as means ± SEM of three observations.

### 2.5. MTT assay

TM6 cells were plated in 12-well plates. Following synchronization, the cells were treated with MSeA and DMSeSe (both at 5 $\mu$M) for 1, 2, and 3 hr. The cells were rinsed once with DMEM:F12 medium (without phenol red, Sigma) and incubated in DMEM:F12 medium (without phenol red) containing MTT at 1 mg/mL for 4 hr at 37° in the dark. The MTT solution was aspirated from the wells, DMSO (3 mL/well) was added, and the optical density was read at 570 nm taking DMSO as the blank [23]. Data are depicted as means ± SEM of three observations.

### 2.6. cDNA array analysis

Treating synchronized TM6 cells with MSeA for 5–15 min showed a significant inhibition in [$^3$H]thymidine incorporation when measured 3 hr later; therefore, we wanted to investigate if an early treatment with MSeA resulted in alteration of gene expression in these cells. For this experiment, synchronized TM6 cells were treated with MSeA (5 $\mu$M) for 10 min, and RNA was isolated from them using TRIzol (Gibco BRL). Untreated cells were included as controls. After treatment with DNase, poly($A^+$) RNA was prepared using the Oligotex mRNA kit (Qiagen). Labeled first strand cDNA probes were prepared from both the MSeA and control poly($A^+$) RNA, and each was hybridized with individual membranes spotted with 588 genes (Clontech, mouse cDNA array). Following washings according to the manufacturer, the membranes were exposed to X-ray film as well as to a PhosphoImager for quantitation. The signals were normalized against $\beta$-actin (housekeeping gene), and the data were represented as fold increase or decrease in expression compared with the untreated control cells.

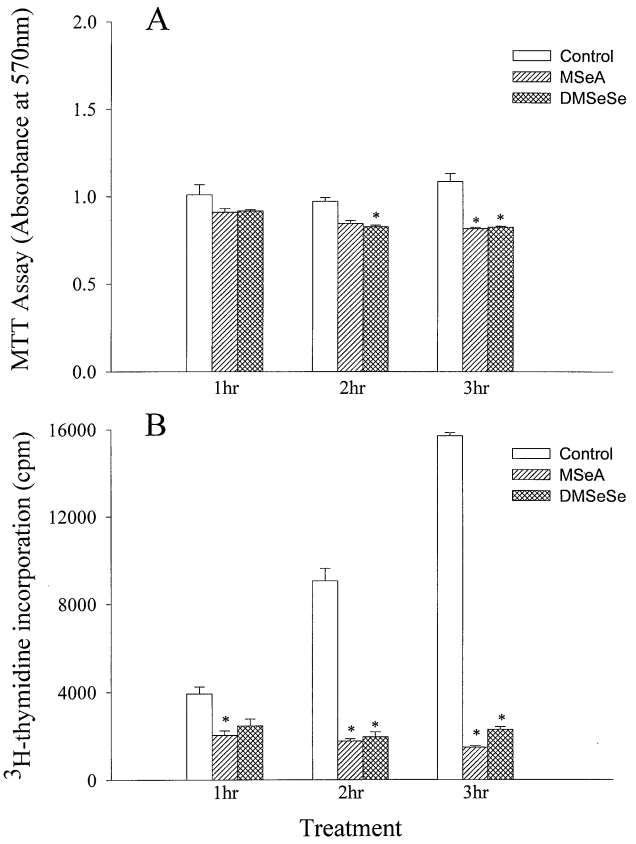


Fig. 2. Effects of MSeA and DMSeSe (5 $\mu$M Se concentration) on the growth of synchronized TM6 cells. (A) MTT assay measuring growth and viability of TM6 cells. (B) [$^3$H]Thymidine incorporation for the DNA synthesis of TM6 cells. Values are means ± SEM; N = 3 at each time point for both assays. Key: (*) indicates significance at $P < 0.05$ when compared with the untreated control.

## 3. Results

Preliminary concentration–response experiments with MSeA demonstrated that 0.001, 0.5, 1, and 5 $\mu$M induced 13, 27, 50, and 81% inhibition in [$^3$H]thymidine incorporation performed after 10 hr of treatment, respectively (data not shown); thus, the experiments described herein were performed using a 5 $\mu$M concentration for all the compounds.

Synchronized TM6 cells treated with either MSeA or DMSeSe showed a decreased optical density in the MTT assay within 2–3 hr of treatment (Fig. 2A) as compared with untreated control cells ($P < 0.05$). [$^3$H]Thymidine incorporation was inhibited significantly ($P < 0.05$) in the cells treated with either 5 $\mu$M MSeA (1, 2, and 3 hr) or DMSeSe (2 and 3 hr) when compared with untreated control cells (Fig. 2B).

The cells were subjected to treatments with MSeA and DMSeSe over a range of time intervals. Cells were treated for 5, 10, 15, 30, 60, 120, and 180 min with either MSeA or DMSeSe (5 $\mu$M), rinsed with incomplete medium, and allowed to incubate with complete medium without Se compounds. A [$^3$H]thymidine incorporation assay was performed for all the treatments and control cells at 3 hr. Both MSeA and DMSeSe inhibited [$^3$H]thymidine incorporation significantly in TM6 cells following 15 min of exposure (Fig. 3).

The synchronized TM6 cells incorporated high levels of [$^3$H]thymidine at the 16-hr time point (Fig. 4A). This corresponds to the maximum number of cells in S phase, as reported earlier [9]. When these cells were allowed to cycle in the presence of MSeA, DMSeS, or DMSeSe, the data showed that these compounds could inhibit [$^3$H]thymidine incorporation (91–97%) up to the 24-hr time point, which is the end of a complete cell cycle (Fig. 4A). Thereafter, it seemed that the compounds had been metabolized completely, as the treated cells resumed proliferation at the 34-hr time point. In fact, these cells were able to achieve the same extent of [$^3$H]thymidine incorporation as untreated

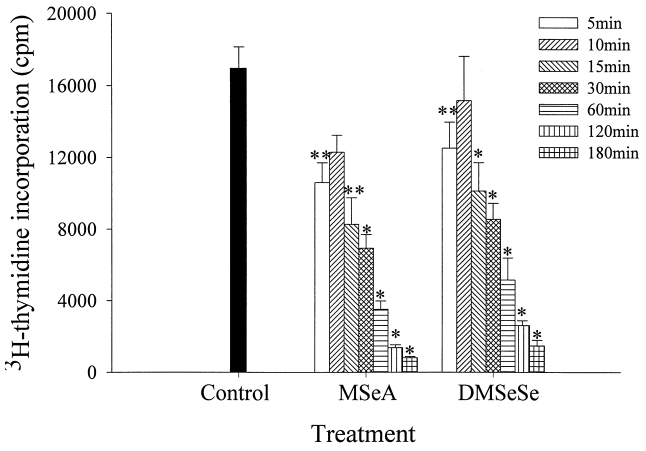


Fig. 3. [$^3$H]Thymidine incorporation in synchronized TM6 cells. Cells were treated with MSeA and DMSeSe (5 $\mu$M Se concentration) for the indicated time duration, and [$^3$H]thymidine incorporation was performed after 3 hr. Untreated TM6 cells served as the control. Values are means ± SEM; N = 3 for each treatment. Key: (**) $P < 0.05$, and (*) $P < 0.01$, compared with the control.

cells. In a separate experiment, DMSS, which is the sulfur analogue of DMSeSe, induced only 9.8% inhibition of growth compared with 80% inhibition induced by DMSeSe (data not shown).

TM6 cells previously treated with MSeA and DMSeSe were exposed to the respective compounds again at the 24-hr time point to investigate if reversibility of the effect was due to loss of chemical over time in culture. The cells exposed to these Se compounds again were inhibited at the 34-hr time point, and started to recover by the 48-hr time point (Fig. 4A). These results confirm that the cells remain sensitive to methylselenol generators.

In another experiment, DMSe and DMSeO, theoretically two inactive metabolites of methylselenol, were compared (Fig. 4B). The synchronized TM6 cells were treated with DMSe and DMSeO at a 6-hr time point, and [$^3$H]thymidine incorporation was measured at 12-, 24-, and 48-hr time points. These compounds, including the solvent methanol, did not inhibit [$^3$H]thymidine incorporation at the 12-hr time point. [$^3$H]Thymidine incorporation was high in the DMSe- and solvent-treated cells at the 24-hr time point but was not statistically significant from the untreated controls. At the 48-hr time point, DMSeO-treated cells showed a slight reduction of 8% ($P < 0.05$) of growth, but this decrease was also observed in the solvent control and was not significant.

The majority of TM6 cells synchronized and treated with 5 $\mu$M MSeA were arrested in the $G_1$ phase of the cell cycle (Fig. 5) with no appreciable apoptosis observed by FACS analysis, using an adherent cell population of MSeA-treated cells. A 10–15% apoptosis, however, was observed in the floating cell population of MSeA (5 $\mu$M)-treated TM6 cells at the 24-hr time point as compared with 5% apoptosis in

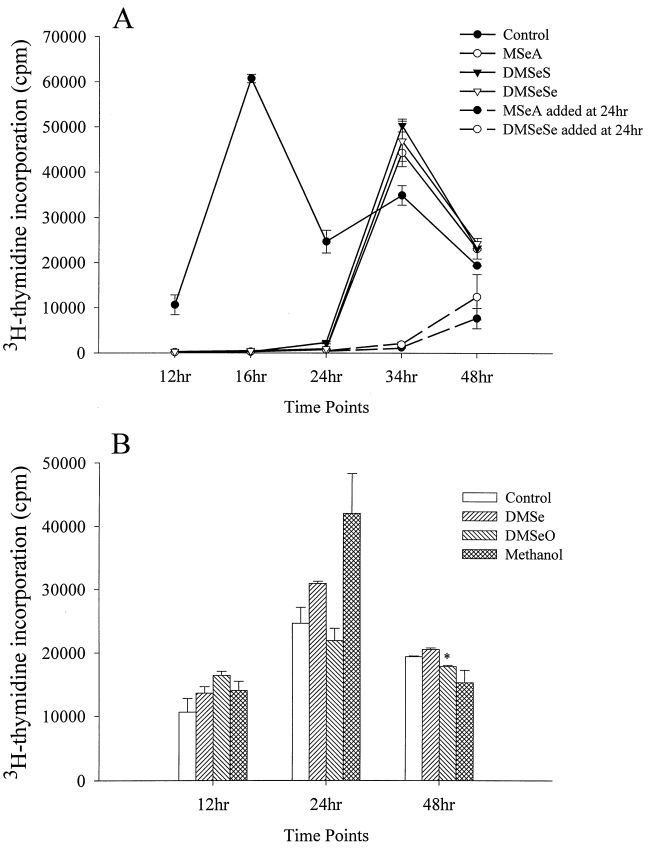


Fig. 4. [$^3$H]Thymidine incorporation in synchronized TM6 cells treated with various Se compounds at the 6-hr time point. (A) Treatments with methylselenol-generating Se compounds (all at a 5 $\mu$M concentration); dashed lines indicate repeat treatment at the 24-hr time point. (B) Treatment with non-methylselenol-generating Se compounds. Methanol was the carrier solvent. Values are means ± SEM, N = 3 for both experiments at each time point. An asterisk (*) indicates $P < 0.05$, compared with untreated TM6 cells that served as controls.

control cells by acridine orange and ethidium bromide staining.[1]

To determine any initial changes occurring at the gene level in the MSeA-treated cells, cDNA array analysis was

[1] Henry Thompson, personal communication.

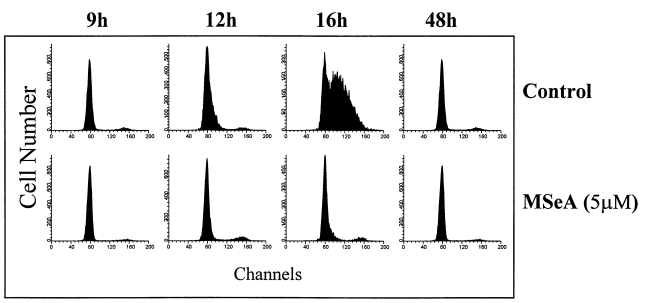


Fig. 5. FACS analysis of MSeA-treated cells. TM6 cells were mainly confined to $G_1$ after treatment with 5 $\mu$M MSeA, with no appreciable apoptosis.

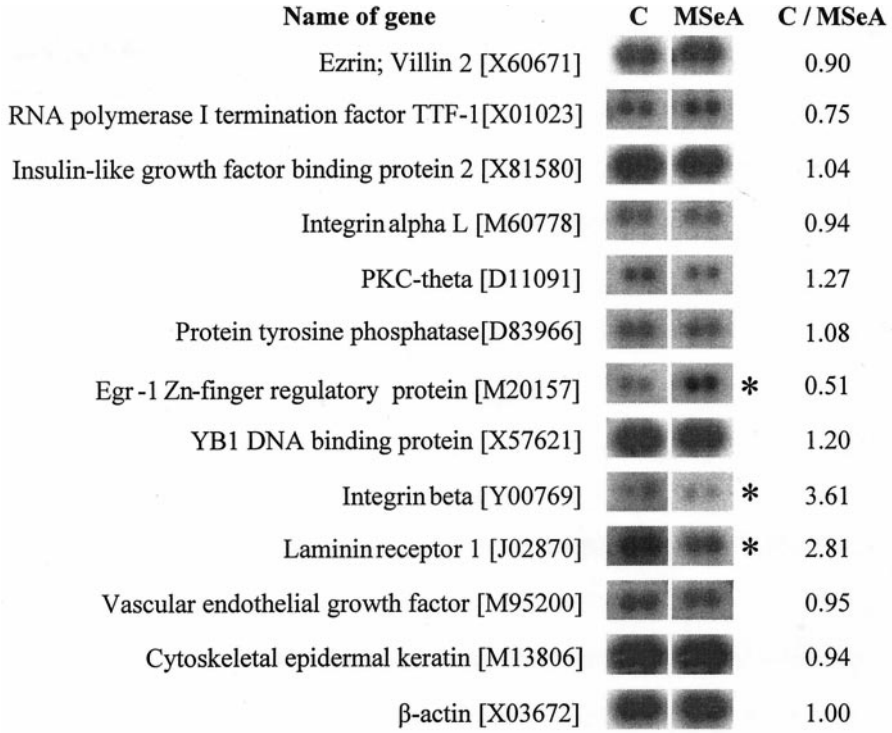


Fig. 6. cDNA array analysis of a 10-min treatment of TM6 cells with 5 $\mu$M MSeA. The three genes marked with an asterisk indicate greater than 2-fold change in expression. $\beta$-Actin (housekeeping gene) was used to normalize the change in fold expression when control (C) and MSeA-treated cells were compared. The numbers in the brackets depict the GenBank Accession Number.

performed using the Atlas mouse cDNA expression array (Clontech). Several genes were differentially expressed in the cDNA array when cells treated for 10 min with MSeA (5 $\mu$M) were compared with control cells. The majority of the cells were in $G_1$ within that given hour (data not shown) and beyond the 9-hr time point (Fig. 5). The differential expression of only three genes was notable. Egr-1, a Zn-finger regulatory protein, was almost 2-fold higher in cells treated with MSeA (Fig. 6), whereas integrin beta and laminin receptor 1 were decreased several fold compared with control cells (Fig. 6). The expression of other genes showed less than 1.3X difference when control and MSeA-treated samples were compared, and these differences were not significant. The expression of several genes including protein tyrosine phosphatase (Accession No. D83966), insulin-like growth factor binding protein 2 (Accession No. X81580), and vascular endothelial growth factor (Accession No. M95200) were not different when control and MSeA treatments were compared (Fig. 6). Of interest was the observation that the screen identified no cell cycle genes, growth factors, or kinases.

## 4. Discussion

The results provide experimental support for the hypothesis of Ip and Ganther [14,15,19] that monomethylated forms of Se are critical effector molecules in Se-mediated growth inhibition *in vitro*. Compounds that were predicted to give rise to methylselenol (MSeA and DMSeSe) were demonstrated to be effective inhibitors of cell growth in synchronized TM6 cells. In contrast, compounds that were predicted to not yield methylselenol (DMSS, DMSeO) were ineffective inhibitors of cell growth. MSC has been reported to be more efficacious than either selenite or selenomethionine in cancer chemoprevention in the range of 1–3 ppm Se [14,15]. Our results show that precursor Se compounds that are able to produce a steady stream of monomethylated metabolite are likely to have good chemopreventive activity.

It is intriguing that a 15-min treatment with MSeA and DMSeSe can significantly inhibit [$^3$H]thymidine incorporation measured 3 hr later. Earlier reports have shown that Se compounds can decrease thymidine kinase activity [24,25]. The [$^3$H]thymidine incorporation in MSeA- and DMSeSe-treated cells remained inhibited until the 24-hr time point, which coincides with the end of a complete TM6 cell cycle, as depicted by the incorporation in control cells. The recovery of cells from the effects of MSeA, DMSeSe, and DMSeS after 34 hr shows that the compounds are no longer interacting with the cellular components of the cell.

After recovery, exposure of the previously treated cells to a fresh concentration of MSeA and DMSeSe again inhibited [$^3$H]thymidine incorporation. Thus, cells that have recovered remain sensitive to methylselenol generators. Although MSC also reduces growth of TM6 cells, inhibition occurs much later at the 48-hr time point as described earlier [9]. Possibly the metabolism of MSC to methylselenol is much slower; therefore, a longer time period is required to

attain a critical level of the methylselenol form. The mechanism of action of MSeA and MSC could be different at the molecular level, and further investigation is needed.

Treatment with a relatively low concentration (5 $\mu$M) of MSeA arrested the TM6 cells, primarily in the $G_1$ phase of the cell cycle. The cells also underwent apoptosis when treated with 50 $\mu$M MSC as reported earlier [9] but only at 48 hr, whereas 50 $\mu$M MSeA could induce apoptosis as early as the 16-hr time point (data not shown). If a high level of methylselenol induces apoptosis, the time difference may be due to slow conversion of the MSC to methylselenol.

Since a rather brief (10–15 min) treatment with MSeA was able to inhibit [$^3$H]thymidine incorporation, we decided to perform cDNA array analysis to determine early changes due to MSeA at the gene level, using the poly($A^+$) prepared from control and MSeA-treated cells. No cell cycle genes, growth factors, or kinases were affected by the treatment. Expressions of two genes (integrin beta and laminin receptor 1) were reduced by a 10-min MSeA treatment at a 5 $\mu$M concentration. These genes are mainly involved in cell attachment. One gene (*Egr-1*) was up-regulated by MSeA treatment. The Egr-1 gene product is a transcription factor with roles in differentiation and growth [26,27]. Egr-1 has been implicated in the induced expression of platelet-derived growth factor (PDGF)-A chain [28], PDGF-B chain [29], TGF$\beta$ [30], and fibronectin [31]. A 45-min MSeA treatment at a 5 $\mu$M concentration in the TM6 cells resulted in a 10-fold induction in Egr-1 expression (cDNA array data not shown) as compared with untreated controls. There were no changes in the PDGF-A and TGF$\beta$ expression at either 10- or 45-min MseA treatments. Further evaluation is required to see if cellular effects of MSeA are associated with any of these genes, especially in a time–course study. The preliminary cDNA array analysis suggests that the effect of MSeA may be on specific protein activity and not ultimately the amount of protein that may be modified. This is supported by the reversibility of the inhibitory effect of MSeA. MSeA can react directly with sulfhydryl groups of proteins or undergo metabolism to other monomethylated forms of Se (as shown in Fig. 1) that can react with sulfhydryl groups or disulfide bonds in target molecules. Possible chemical reactions relevant to the regulation of cellular metabolism by monomethylated Se derivatives are described elsewhere [32]. In summary, MSeA is useful for the direct provision *in vitro* of highly reactive monomethylated Se metabolites that are implicated in Se inhibition of tumor cell growth and other chemopreventive mechanisms.

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