Research Article

In vitro growth inhibition of mouse mammary epithelial tumor cells by methylseleninic acid: Involvement of protein kinases

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Methylseleninic acid (MSeA) is a synthetic organoselenium form known to be effective against mammary carcinogenesis *in vivo*. Using the synchronized mouse mammary epithelial tumor cell (TM6) model, we have previously shown that 5 μM MSeA significantly inhibits cell growth and induces a reversible growth arrest in the G1 phase. In the present study, we examined the effects of MSeA on Rb, cyclin dependent kinase 2 (cdk2), cdk4, cyclin E and cyclin D1. Growth arrest of cells was accompanied by a reduction in total cdk2 kinase and cyclin E-associated cdk2 kinase activities. The p27 levels associated with cdk2 were elevated during the cell cycle. In addition, growth inhibition correlated with a relative increase in the hypophosphorylated form of Rb in MSeA-treated cells and Egr1 was elevated in MSeA-treated cells. The Kinetworks™ Protein Kinase Screen (KPKS 1.0) was used to examine 75 protein kinases. MSeA treatment resulted in differential expression of several protein–serine/threonine kinases, protein–tyrosine kinases and protein–threonine/tyrosine kinases. Some of these kinases are being reported for the first time as being altered by MSeA. The outcome of these experiments will be of significance since these kinases are known to be involved in survival and/or apoptotic pathways of tumor cells.

Keywords: KPKS-1.0 / Mammary epithelial tumor cells / Methylseleninic acid / Protein kinases / Synchronized cells

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1 Introduction

Several organoselenium compounds have been screened for their chemopreventive potential against the development of tumors in mammary gland, lung, colon, and prostate [1–5], and recent studies indicate that supplemental selenium reduces cancer risk in humans [6]. The chemopreventive ability of selenium as an anticancer agent depends on its chemical form and the metabolism of the parent compound is critical to provide the reactive form [7]. It has long been hypothesized that simple monomethyl form of selenium should have strong growth inhibitory activity at low concentration and be able to exert the effect rapidly [7, 8]. Semethylselenocysteine (MSC) is a good precursor for generating monomethylated selenium as it can be converted directly to methylselenol (CH_3SeH) *via* a cysteine conjugate β-lyase reaction [9]. To study the mechanisms of selenium's anticancer action, an oxidized monomethylated form of selenium, methylseleninic acid (MSeA), is being used as a direct precursor for CH_3SeH [10, 11] which has been shown to be effective against mammary carcinogenesis *in vivo* [11]. Previously we had demonstrated that treatment of mouse mammary epithelial tumor (TM6) cells with 5 μM MSeA for 10–15 min was sufficient to inhibit DNA synthesis in these cells followed by a growth arrest in G1 phase [10].

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Abbreviations: cdk2, cyclin dependent kinase 2; **COT,** cancer Osaka thyroid oncogene; **ERK,** extracellular-regulated kinase 1/2; **FAK,** focal adhesion kinase; **FYN,** Fyn oncogene related to SRC; **GRK2,** G protein-coupled receptor kinase 2; **HPK1,** hematopoietic progenitor kinase 1; **MEK,** mitogen activated protein kinase kinase; **MSeA,** methylseleninic acid; **NAF,** sodium fluoride; **PSTK,** protein–serine/threonine kinase; **PYK,** protein–tyrosine kinase; **PTYK,** protein–threonine/tyrosine kinase; **RSK1,** ribosomal S6 kinase; **SAPKβ**, stress-activated protein kinase β; **S6Kp70,** S6 kinase p70; **SYK,** spleen tyrosine kinase; **TCA,** trichloroacetic acid

The DNA replication and mitosis are controlled by the activation of S phase- and M-phase specific cyclin-dependent protein kinases (cdks), respectively. The catalytic subunits of these kinases are only active when complexed with their specific regulatory subunits termed cyclins. In mammalian cells S phase is controlled by cyclin dependent kinase 2 (cdk2) complexed with cyclins E and A and the M phase is regulated by cdc2 associated with cyclins A and B. Mammalian cells also have G1-specific cdks and cyclins that promote the synthesis of proteins needed for chromosome duplication and trigger activation of S phase cdks [12 – 14].

The present study was designed to examine the effects of MSeA on several cell cycle regulators including Rb, cdk2, cdk4, cyclin E, and cyclin D1. In particular total cdk2 kinase and cyclin E-associated cdk2 kinase activities were measured along with the p27 levels associated with cdk2 as a consequence of MSeA treatment in synchronous population of TM6 cells. Furthermore, we utilized the Kinetworks Protein Kinase Screen (KPKS 1.0) to investigate the involvement of several protein kinases in selenium-mediated mammary growth inhibition.

2 Materials and methods

2.1 Agents, cell culture, and synchronization

MSeA was purchased from PharmaSe (Lubbock, TX). The TM6 tumor cell line was originally derived from the COMMA-D mouse mammary epithelial cells [15]. TM6 cells were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5 μg/mL insulin (Sigma, St. Louis, MO), 5 ng/mL epidermal growth factor (EGF) (Sigma), 2% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), and 1% penicillin – streptomycin (Invitrogen) at 37°C in a humidified atmosphere in the presence of 5% CO_2. For treatments, TM6 cells were seeded at 6.6×10^3 cm^{-2} in either 100 mm culture dishes or 12-well plates and allowed to grow for 48 h in regular medium as described above. Cells were synchronized by starvation in minimal medium (DMEM/F12 medium without growth factors and serum) as described earlier [16]. TM6 cells were released from growth arrest by feeding them regular medium (DMEM/F12 supplemented with growth factors and serum) for 6 h at which time one set of cells was treated with 5 μM MSeA. Cells were collected at 9, 12, and 24 h after feeding them with regular medium and subjected to the following assays. Untreated cells at these time points served as controls.

2.2 ^{3}H-thymidine incorporation

TM6 cells were grown in 12-well plates (2.5×10^4 cells/well). Synchronized cells were treated with 5 μM MSeA and the ^{3}H-thymidine incorporation assay was performed after the 9, 12, 24, 34, and 48 h time points as described earlier [16]. Briefly, TM6 cells were rinsed twice with fresh medium following the treatment with MSeA and pulsed with 1 μCi ^{3}H-thymidine for 1 h at 37°C. Cells were rinsed thrice each with 20 mM Tris-HCl (pH 7.5), 10% trichloroacetic acid (TCA) solution, and finally the precipitates were dissolved in 0.5 mL 1 N NaOH for 30 min on a rotator-shaker. Each well was counted in a liquid scintillation counter and the data were depicted as percentage increase or decrease in incorporation of radiolabeled thymidine as compared to the untreated control cells at each time point (control incorporation was taken as 100% uptake). All determinations were performed in triplicate and statistically analyzed by Student's *t*-test.

2.3 Cdk2 and Cdk4 kinase assays

TM6 cells (5.2×10^5) were seeded in each 100 mm culture dish. Following treatment with 5 μM MSeA, the cells were washed in 1X PBS and processed for either cdk2 or cdk4 kinase assays. To prepare lysates for cdk2 kinase assay, cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EGTA, protease inhibitor mixture (leupeptin (50 μg/mL), 1 mM sodium orthovanadate, 1 mM PMSF, aprotonin (25 U/mL), 1 mM sodium fluoride (NaF), and 10 mM β-glycerophosphate) for 15 min on ice. The cells were lysed in buffer B for cdk4 kinase assay (50 mM HEPES, pH 8.0, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 2.5 mM EGTA, 1 mM EDTA) and protease inhibitor mixture (as described above) for 15 min on ice. The cell suspensions for each assay were further sonicated and centrifuged at 40 000 rpm for 1 h at 4°C. Supernatants were measured for their protein content by Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Each sample was precleared and the proteins were immunoprecipitated using rabbit polyclonal cdk2 or cdk4 (Santa Cruz Biotechnology, Santa Cruz, CA) 5 μg/500 μg sample protein for the cdk2 and cdk4 kinase assays as described earlier [16]. For cdk2, Histone H1 (Boehringer-Mannheim, Mannheim, Germany) and for cdk4, recombinant Rb (QED Bioscience, San Diego, CA) was used as substrate. The bands appearing at 30 – 34 and 110 kDa regions were scanned using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX) and the optical densities (integrated intensity in arbitrary units) of the control and MSeA-treated samples were compared for cdk2 and cdk4 kinase activities for all time points. The assays were performed twice for all the samples.

2.4 Isolation of cytosolic and nuclear extracts

TM6 cells were washed 2 times in PBS and subsequently suspended in 0.3 mL nuclear buffer (consisting of 2 mM $MgCl_2$, 5 mM K_2HPO_4, 0.1 mM EDTA, and protease inhibitor mixture as described above). An additional 0.3 mL nuclear buffer (containing 0.7% Triton X-100) was added

after the tubes had been incubated on ice for 8 – 10 min. The suspensions were centrifuged at 800 rpm for 10 min at 4°C; the pellets were further subjected to lysis for obtaining nuclear fractions. The supernatants were centrifuged at 40 000 rpm for 1 h and designated as cytosolic fraction. The nuclear pellets were washed once with nuclear buffer and the nuclear extracts were prepared by resuspending the pellet in 0.3 mL buffer containing 20 mM HEPES (pH 7.8), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.1 mM Na_2VO_3, 50 mM NaF, 20 μg/mL leupeptin, 20 μg/mL aprotonin, and 5 mM β-glycerophosphate. Sonication was carried out on ice using the Ultrasonic processor, and the mixtures were examined microscopically for complete break of the nuclei. The mixtures were centrifuged at high speed and the supernatants were designated as nuclear extracts and stored at –20°C after determining the protein content of the cytosolic and the nuclear extracts as described above.

2.5 Kinase assay for cyclin E – cdk2 complex

Nuclear fractions (200 μg protein) were precleared with normal goat serum and immunoprecipitated with anti-cyclin E antibody in the presence of protein A sepharose beads. The immune complexes were processed for cdk2 kinase assay as described above.

2.6 Expression of cyclin E and p27 bound to cdk2

Nuclear fractions (200 μg) were precleared and immunoprecipitated with anti-cdk2 antibody (UBI, Waltham, MA) in the presence of protein-A sepharose beads. The immune complexes were washed thrice with lysis buffer (without inhibitors) and resuspended in 20 μL of solubilization buffer, boiled for 5 min, and separated on 10% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membrane, blocked with 5% milk in TBST for 1 h incubated with anti-cyclin E antibody (1 μg/mL) (Santa Cruz Biotechnology), and anti-p27 antibody (1:2000) for overnight on a rocker at slow speed. The blots were washed in TBST (thrice, 10 min each) and incubated with antirabbit-HRP at 1:3000 dilution (for cyclin-E) and with antimouse-HRP at 1:3000 dilution (for p27) for 1 h at room temperature. The blots were washed thrice in TBST as described above followed by a wash in TBS. The reactions were developed using ECL detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ).

2.7 Western blotting for Egr1, cyclin D1, Rb, p27, and cleaved-PARP

Protein (50 μg) from total cell lysates (for cyclin D1, D3, p27, and cleaved-PARP) was electrophoresed on 10% SDS-PAGE and 50 μg from nuclear extracts (for Rb) was electrophoresed on 7.5% SDS-PAGE. These gels were electrotransferred separately onto nitrocellulose paper, blocked with 5% fat free dry milk in Tris-buffered saline (0.1 M, pH 7.5) containing 0.1% Tween-20 (TBST) for 1 h. Anti-cyclin D1 and anti-cyclin D3 antibodies (1 μg/mL) (UBI), anti-Rb antibody (1:2000) (Pharmingen, San Diego, CA), anti-p27 antibody (1:2000) (BD Biosciences, San Diego, CA) and cleaved-PARP antibody (1:1000) (Cell Signaling Technology, Danvers, MA) were added to the blots in 5% milk-TBS (0.1 M, pH 7.5) for overnight at 4°C on a rocker platform. The blots were washed thrice in TBST for 10 min each, followed by incubation with either anti-mouse or anti-rabbit-HRP labeled antibody at a dilution of 1:3000 in 5% milk-TBST for 1 h at RT. Blots were washed thrice in TBST for 5 min each followed by a final wash in TBS. The antibody reactions were revealed using the ECL detection reagents (GE Healthcare Bio-Sciences).

2.8 Screening for phospho-kinases

TM6 cells (5.2×10^5) were seeded in each 100 mm culture dish. Following treatment with 5 μM MSeA, the cells were washed in 1 × PBS, lysed by adding 0.5 mL lysis buffer (20 mM MOPS; pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM Na_2VO_3, 1 mM PMSF, 3 mM benzamidine, 5 mM pepstatin, 10 μM leupeptin, and 0.5% Triton X-100; final pH 7.0) for 5 min at 4°C. Cells were sonicated twice for 15 s and the homogenate was subjected to ultracentrifugation for 30 min at 50 000 rpm. The protein concentration of the supernatant fraction was measured as described above and phosphokinase screening was performed by Kinexus (Vancouver, BC; analyses KPKS-1.0).

3 Results and discussion

3.1 Effect of MSeA on DNA synthesis

MSeA is a potent growth inhibitor of mammalian epithelial tumor cells *in vitro* [10, 11] and *in vivo* [11] but its mechanism of action at molecular level needs further investigation. Our previous investigation showed that a short exposure of as low as 15 min could result in inhibition of mouse mammary epithelial tumor cell growth [10]. A synchronized model of TM6 tumor cells was used in the present study to extend these observations by examining specific changes associated with MSeA treatment to trigger the specific events during cell cycle. The synchronized TM6 cells treated with MSeA were compared for their ^{3}H-thymidine incorporation to the untreated control cells (Table 1). MSeA treatment of these lowered the ^{3}H-thymidine incorporation in comparison to control cells beginning at the 9 h time point ($p < 0.05$). The inhibitory effect of MSeA was further amplified at 12 h (6.4% of control cells, $p < 0.001$) and 24 h (1.5% of control cells, $p < 0.001$). The decrease in ^{3}H-thymidine incorporation paralleled the increase in

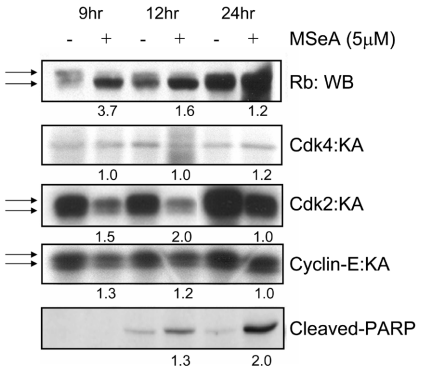

Figure 1. Effect of MSeA on cell cycle regulatory proteins in synchronized TM6 cells. TM6 cells were synchronized and treated with 5 μM MSeA for different time points as described in Section 2. pRB Western blot, kinase activities (KA) for cdk2, cdk4, and cyclin E-associated and PARP cleavage was performed as described in Section 2. Fold changes in MSeA treated samples are indicated below respective assays as compared to controls.

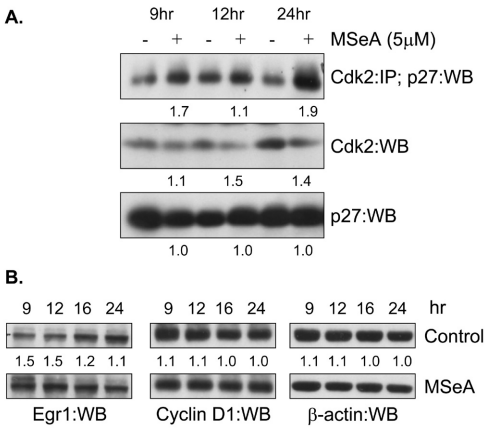

Figure 2. Effect of MSeA on cdk2-associated p27 in TM6 nuclear fractions. Synchronized TM6 cells were treated with 5 μM MSeA as described in Section 2. (A) cdk2 was immunoprecipitated and probed with p27. Proteins were also probed with p27 and cdk2 alone. Note the increase in p27 associated cdk2 levels. (B) Egr1 and cyclin D1 protein levels following MSeA treatment. Note the increased levels of Egr1 in early time points.

cleaved-PARP levels (Fig. 1), indicative of apoptosis in MSeA-treated TM6 cells starting at 12 h. The MSeA-treated TM6 cells begin to incorporate ^{3}H-thymidine equivalent to untreated controls at the 34 h time point; indicating that the MSeA is used up and therefore no longer able to inhibit growth. There was no PARP-cleavage (data not shown) in the cells at this stage. When the synchronized TM6 cells were treated with 5 μM MSeA again at the 24 h time point, these cells responded in growth inhibition, illustrating their continued sensitivity to the fresh exposure of MSeA [10].

3.2 Effect of MSeA on Rb expression

Rb was hypophosphorylated at 9 h time point in the MSeA-treated cells (increased by 3.7-fold) and remained increased at the 12 h time point (increased by 1.6-fold) when compared to untreated controls. By the 24 h time point both the control and MSeA-treated cells had similar levels of total Rb (Fig. 1). The total E2F1 levels however, did not change because of MSeA treatment during the cell cycle (data not shown). pRb is the master switch regulating cell cycle progression, and its continuing phosphorylation parallels cell transit through G1 and S phase [17, 18]. Our results depict the hyperphosphorylated pRb in control cultures in progression through cell cycle with no hypophosphorylated pRb. However, exposure of TM6 cells to MSeA restored, to a large degree, the active mode of pRb as evidenced by increased expression of hypophosphorylated pRb in 9 and 12 h time point concomitant with the decrease in overall cell proliferation. These results are consistent with some of the recent reports [19, 20] showing association of cell cycle arrest with decreased pRb phosphorylation.

3.3 Effect of MSeA on cdk4 kinase activity and cyclin D1 levels

The cdk4 kinase activity measured by monitoring the intensity of 40 kDa Rb band was unchanged during TM6 cell cycle following MSeA treatment (Fig. 1). Cdk4 kinase activity is associated mainly with cyclin D1, which functions to regulate transit through early G1 phase. The present data did not show any change in cyclin D1 as a result of MSeA treatment (Fig. 2B). However, in a previously published study using TM6 hyperplastic cells MSeA was reported to decrease cyclin D1 [20]. This discrepancy can be explained on the basis of time point selection for measurement of cyclin D1 and the use of TM6 hyperplastic cells used in the reported study [20].

3.4 Effect of MSeA on total cdk2 and cyclin E-associated kinase activities

The total cdk2 kinase activities were decreased following MSeA treatment by more than 1.5-fold at 9 h and this decrease was further evident at the 12 h time point (2.0-fold). The MSeA-treated cells had recovered at 24 h time point (1.0-fold) when compared to untreated controls (Fig. 1). The present data on decreased phosphorylation of cdk2 correlates with the time frame when ^{3}H-thymidine incorporation is inhibited in these cells. The cdk2 kinase activity measured in samples that were immunoprecipitated with anti-cyclin E antibody also revealed the same trend as seen for the total cdk2 kinase activity at the 9 h (reduction by 1.3-fold) and 12 h (reduction by 1.2-fold) as compared to untreated controls. At the 24 h time point no change was noted in the cyclin E-associated cdk2 kinase activity in the

Table 1. Effect of MSeA (5 μM) on TM6 cell proliferation as measured by DNA synthesis

Time point (h)	^{3}H-Thymidine incorporation (cpm) Control	SeA
9	541 ± 118	138 ± 13.7*
12	991 ± 188	63.6 ± 7.8**
24	3888 ± 162	59.6 ± 12.2**
48	4176 ± 1337	4778 ± 190

Values are Mean ± SE of three observations. The data were analyzed by Student's *t*-test between control and MSeA-treated cells at each time point. $p < 0.05$ (*), $p < 0.001$ (**).

MSeA-treated cells when compared with untreated controls. (Fig.1).

3.5 Effect of MSeA on cdk2-associated p27

The immunoprecipitation of cdk2 followed by Western blot analysis of associated cyclin E showed no changes following MSeA treatments at 9 and 12 h time points (data not shown), indicating that the alteration in cdk2 kinase activity might be due to either altered phosphorylation or the presence of cyclin inhibitors. Hence, this led us to examine the level of expression of two direct inhibitors of cdk activity [21], which includes p21and p27. p21 is known to accumulate in response to DNA damage in a p53-dependent manner and can be induced in a p53-independent fashion [22]. p21 induction by MSeA in p53 null cells has been reported [23]. In TM6 cells however, p21 was not detected (data not shown) probably because of mutated p53 in TM6 cells. Thus, p27 became our focus of study. The protein expression for p27 bound to cdk2 increased at 9, 12, and 24 h time points (Fig. 2A) while the total p27 protein levels remained unchanged at all time points and cdk2 levels were slightly reduced at 12 and 24 h time points (Fig. 2A). This observation, in addition to the decreased cdk2 kinase activity at the corresponding time points (Fig. 1), suggests that MSeA treatment results in increased association of p27 to cdk2 leading to a decrease in its kinase activity without altering the total p27 pool.

3.6 Effect of MSeA on Egr1

The expression levels Egr1 were elevated during TM6 cell cycle following MSeA treatment (Fig. 2B). These data confirm our previous finding of increased expression of *Egr1* gene [10] and are suggestive of a possible role of MSeA in enhancing a tumor suppressor in mammary epithelial tumors [24].

3.7 Effect of MSeA on phospho-kinases

In the synchronized TM6 cells, 51 phosphokinases were detected out of the possible 75 phosphokinases tested in the

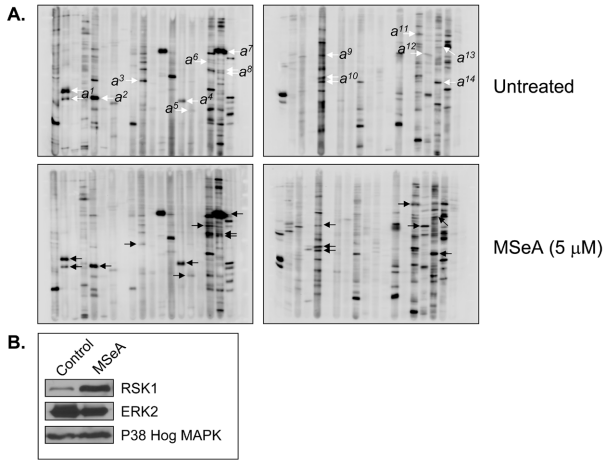

Figure 3. (A) Discovery of selenium-responsive protein kinases using phospho-site screening in synchronized TM6 cells. Synchronized TM6 cells were incubated with 5 μM MSeA and analyzed by the Kinetworks Protein Kinase Screen (KPKS-1.0) screening system at 24 h time point. The identities of protein targets are indicated by arrows a^1: ERK1/2 [PSTK], a^2: p38 Hog MAPK [PSTK], a^3: SAPKb [PSTK], a^4: MEK4 [PTYK], a^5: MEK6 [PTYK], a^6: S6Kp70 [PSTK], a^7: RSK1 [PSTK], a^8: COT [PSTK], a^9: SYK [PYK], a^{10}: FYN [PYK], a^{11}: FAK [PYK], a^{12}: GRK2 [PSTK], a^{13}: HPK1 [PSTK], a^{14}: ZIP Kinase [PSTK]. PSTK: protein–serine/threonine kinase, PTYK: protein–threonine/tyrosine kinase, PYK: protein–tyrosine kinase. (B) Confirmation of selected kinases from KPKS screen in MSeA-treated TM6 cells. Synchronized TM6 cells were treated with 5 μM MSeA and lysates at 24 h time point were investigated for RSK1, p38 Hog MAPK, and ERK2 expression.

KPKS 1.0 screen (Table 2). Following treatment with 5 μM MSeA treatment at 24 h time point seven phosphokinases including; cancer Osaka thyroid oncogene (COT), G protein-coupled receptor kinase 2 (GRK2), hematopoietic progenitor kinase 1 (HPK1), mitogen activated protein kinase kinase (MEK4), MEK6, ribosomal S6 kinase 1 (RSK1), and S6 kinase p70 (S6Kp70) were upregulated as compared to untreated TM6 cells. While seven phosphokinases were decreased after MSeA treatment in comparison to untreated TM6 cells, these are extracellular-regulated kinase 1 (ERK1), ERK2, focal adhesion kinase (FAK), Fyn oncogene related to SRC (FYN), p38 Hog MAPK, spleen tyrosine kinase (SYK), stress-activated protein kinase β (SAPKβ), and ZIP Kinase (Fig. 3A, Table 2). Changes in several kinases following MSeA treatment of TM6 cells suggests that multiple pathways are affected by selenium. Western blot analysis for selected kinases; RSK1, p38 Hog MAPK, and ERK2 confirmed the data from the KPKS screen (Fig. 3B). Akt and p38MAPK in the present study were reduced in MSeA-treated TM6 cells, these data are in support of the previously published study correlating a decrease in ERK1/2 kinases [25]. FAK is a cytoplasmic protein–tyrosine kinase (PYK) that promotes cell migration, survival, and gene expression and a decreased expression of

Table 2. Kinetworks Protein Kinase Screen (KPKS-1.0) in TM6 mouse mammary epithelial tumor cells following treatment with 5 μM MSeA. The data are means of three different assays and are an estimate of blot density. Protein kinases that were undetected are given a value of "0." Average mean ratios of values in Control/MSeA are indicated. Protein kinases followed by letter "a" and a number correspond to the data in Western immunblot screen in Fig. 3A

Full name of protein	Abbreviation	Type	Control Trace Qty	MSeA Trace Qty	C/MSeA
Bone marrow X kinase	BMX	PSTK	10893	8753	0.80
Bruton agammaglobulinemia tyrosine kinase	BTK	PYK	0	0	
Calmodulin-dependent kinase kinase	CaMKK	PSTK	9522	4408	2.16
Calmodulin-dependent kinase 1	CaMK1	PSTK	0	0	
Calmodulin-dependent kinase 4	CaMK4	PSTK	0	5225	
Cyclin-dependent kinase 5	CDK5	PSTK	12399	16470	0.75
Cyclin-dependent kinase 6	CDK6	PSTK	6695	7631	0.88
Cyclin-dependent kinase 7	CDK7	PSTK	13213	10862	1.22
Cyclin-dependent kinase 9	CDK9	PSTK	0	0	
Casein kinase 1δ	CK1d	PSTK	7900	6759	1.17
Casein kinase 1ε	CK1e	PSTK	0	0	
Casein kinase 2a	CK2a	PSTK	15061	21130	0.71
Casein kinase 2b	CK2b	PSTK	8255	16783	0.49
Casein kinase 2c	CK2c	PSTK	8441	9798	0.86
Cancer Osaka thyroid oncogene (Tpl2) *a*[8]	COT	PSTK	8249	26486	0.26
c-SRC tyrosine kinase	CSK	PYK	8459	4639	1.82
Death associated protein kinase 1	DAPK	PSTK	0	0	
DNA-activated protein kinase	DNAPK	PSTK	0	0	
Extracellular-regulated kinase 1 *a*[1]	ERK1	PSTK	36223	30639	1.18
Extracellular-regulated kinase 2 *a*[1]	ERK2	PSTK	17374	17029	1.02
Extracellular-regulated kinase 3	ERK3	PSTK	10132	0	
Extracellular-regulated kinase 6	ERK6	PSTK	0	0	
Focal adhesion kinase *a*[11]	FAK	PYK	16314	12208	1.34
Fyn oncogene related to SRC *a*[10]	FYN	PYK	16987	12589	1.35
Germinal center kinase	GCK	PSTK	0	5956	
G protein-coupled receptor kinase 2 *a*[12]	GRK2	PSTK	11613	11866	0.98
Glycogen synthase kinase 3α	GSK3a	PSTK	20889	27055	0.77
Glycogen synthase kinase 3β	GSK3b	PSTK	0	13991	
Hematopoietic progenitor kinase 1 *a*[13]	HPK1	PSTK	11899	14785	0.80
Inhibitor NF-kB kinase α	IKKa	PSTK	9800	9913	0.99
Janus kinase 1	JAK1	PTK	15111	15690	0.96
Janus kinase 2	JAK2	PTK	0	2773	
Kinase suppressor of Ras 1	KSR1	PSTK	0	0	
Lymphocyte-specific protein tyrosine kinase	LCK	PYK	0	0	
MAP kinase kinase 1 (MKK1)	MEK1	PTYK	0	6420	
MAP kinase kinase 2 (MKK2)	MEK2	PTYK	10372	17043	0.61
MAP kinase kinase 4 (MEK4) *a*[4]	MEK4	PTYK	17539	28406	0.62
MAP kinase kinase 6 (MEK6) *a*[5]	MEK6	PTYK	8379	22269	0.38
MAP kinase kinase 7 (MEK7)	MEK7	PTYK	3856	0	
MAP kinase interacting kinase 2	MNK2	PSTK	0	0	
Mammalian sterile 20-like 1	MST1	PYK	0	0	
NIMA (never in mitosis)-related kinase 2	NEK2	PSTK	0	0	
Oncogene Lyn	LYN	PYK	0	5663	
Oncogene Raf 1	RAF1	PSTK	21976	12772	1.72
Oncogene SRC	SRC	PYK	5295	3853	1.37
p21-activated kinase 1 (PAKα)	PAK1	PSTK	0	0	
p21-activated kinase 3 (PAKβ)	PAK3	PSTK	0	0	
p38 Hog MAP kinase *a*[2]	38 MAPK	PSTK	40744	32978	1.24
3-Phosphoinositide-dependent protein kinase 1	PDK1	PSTK	0	0	
Pim1	PIM1	PSTK	16708	16830	0.99
Protein kinase A (cAMP-dependent protein kinase)	PKA	PSTK	0	0	
Protein kinase Bα (Akt1)	PKBa	PSTK	8211	4430	1.85
Protein kinase Cα	PKCa	PSTK	8449	9717	0.87
Protein kinase Cβ1	PKCb	PSTK	12661	13172	0.96
Protein kinase Cδ	PKCd	PSTK	7062	13257	0.53
Protein kinase Cε	PKCe	PSTK	4568	6469	0.71
Protein kinase Cγ	PKCg	PSTK	5189	0	
Protein kinase Cλ	PKCl	PSTK	10530	13538	0.78

Table 2. Continued

Full name of protein	Abbreviation	Type	Control Trace Qty	MSeA Trace Qty	C/MSeA
Protein kinase Cμ	PKCm	PSTK	0	0	
Protein kinase Cθ	PKCt	PSTK	0	0	
Protein kinase Cζ	PKCz	PSTK	39949	56739	0.70
Protein kinase G1 (cGMP-dependent protein kinase)	PKG1	PSTK	0	0	
Protein tyrosine kinase 2	PYK2	PYK	0	0	
dsRNA-dependent kinase	PKR	PSTK	0	0	
RhoA kinase	ROKa	PYK	0	0	
Ribosomal S6 kinase 1 *a*[7]	RSK1	PSTK	55190	93532	0.59
Ribosomal S6 kinase 2	RSK2	PSTK	0	0	
S6 kinase p70 *a*[6]	S6Kp70	PSTK	6744	20395	0.33
Spleen tyrosine kinase *a*[9]	SYK	PYK	33202	8060	4.12
Stress-activated protein kinase β *a*[3]	SAPKb	PSTK	21321	12526	1.70
v-mos Moloney murine sarcoma viral oncogene homolog	MOS	PSTK	7309	10748	0.68
v-raf Murine sarcoma viral oncogene homolog B1	RAFB	PSTK	9103	0	
Yamaguchi sarcoma viral oncogene	YES	PYK	0	4149	
ζ-Chain (TCR) associated protein kinase	ZAP70	PYK	0	0	
ZIP kinase (death associated protein kinase 3) *a*[14]	ZIP	PSTK	21702	21499	1.01

FAK following MSeA treatment may be a contributing factor to TM6 growth inhibition. Moreover, a reduced expression in FAK and FYN by MSeA may play a role in growth inhibition of TM6 cells as both these kinases in breast carcinomas correlate with shorter survival [26]. Furthermore, elevation of S6Kp70 by selenite has been reported earlier [27] and our data correlate with this report. A detailed investigation is required to integrate the signaling networks associated with the several kinases altered by MSeA treatment of TM6 cells.

4 Concluding remarks

MSeA, a synthetic organoselenium compound is a potent inhibitor of mouse mammary epithelial tumor cell growth and involves alteration of several protein kinases including cdk2 and other novel kinases reported here for the first time to initiate interest in this field. The several kinases reported here need to be investigated, preferably, in a synchronized model to assign cell cycle specific regulatory roles in MSeA-induced growth inhibition.

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5 References

[1] Ip, C., Ganther, H. E., Comparison of selenium and sulfur analogs in cancer prevention. *Carcinogenesis* 1992, *13*, 1167 – 1170.

[2] Lu, J. X., Jiang, C., Kaeck, M., Ganther, H. E., Cellular and metabolic effects of triphenylselenonium chloride in a mammary cell culture model. *Carcinogenesis* 1995, *16*, 513 – 517.

[3] Das, A., Desai, D., Pittman, B., Amin, S., *et al*., Comparison of the chemopreventive efficacies of 1,4-phenylenebis(methylene)selenocyanate and selenium-enriched yeast on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced lung tumorigenesis in A/J mouse. *Nutr. Cancer* 2003, *46*, 179 – 185.

[4] Reddy, B. S., Revenson, A., Kulkarni, N., Upadhayaya, P., *et al*., Chemoprevention of colon carcinogenesis by the synthetic organoselenium compound 1,4-phenylene bis(methylene)selenocyanate. *Cancer Res*. 1992, *52*, 5635 – 5640.

[5] Lee, S. O., Yeon Chun, J., Nadiminty, N., Trump, D. L., *et al*., Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *Prostate* 2006, *66*, 1070 – 1705.

[6] Clark, L. C., Combs, G. F., Jr., Turnbull, B. W., Slate, E. H., *et al*., Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA* 1996, *276*, 1957 – 1963.

[7] Ip, C., Ganther, H. E., in: Wattenberg, L., Lipkin, M., Boone, C. W., Kelloff, G. J. (Eds.), *Cancer Chemoprevention*, CRC Press, Boca Raton, FL 1992, pp. 479 – 488.

[8] Ip, C., Ganther, H. E., in: Burk, R. F. (Ed.), *Selenium and Biology and Human Health*, Springer-Verlag, New York 1993, pp. 171 – 180.

[9] Rooseboom, M., Vermeulen, N. P., van Hemert, N., Commandeur, J. N., Bioactivation of chemopreventive selenocysteine Se-conjugates and related amino acids by amino acid oxidases novel route of metabolism of selenoamino acids. *Chem. Res. Toxicol*. 2001, *14*, 996 – 1005.

[10] Sinha, R., Unni, E., Ganther, H. E., Medina, D., Methylseleninic acid, a potent growth inhibitor of synchronized mouse mammary epithelial tumor cells in vitro. *Biochem. Pharmacol*. 2001, *61*, 311 – 317.

[11] Ip, C., Thompson, H. J., Zhu, Z., Ganther, H. E., In vitro and in vivo studies of methylseleninic acid: Evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. *Cancer Res.* 2000, *60*, 2882 – 2886.

[12] Sherr, C. J., Cancer cell cycles. *Science* 1996, *274*, 1672 – 1677.

[13] King, R. W., Deshaies, R. J., Peters, J. M., Kirschner, M. W., How proteolysis drives the cell cycle. *Science* 1996, *274*, 1652 – 1659.

[14] Stillman, B., Cell cycle control of DNA replication. *Science* 1996, *274*, 1659 – 1664.

[15] Jerry, J. D., Butel, J. S., Medina, D., p53 mutations in Comma-D cells. *In Vitro Cell. Dev. Biol*. 1994, *30*, 87 – 89.

[16] Sinha, R., Medina, D., Inhibition of cdk2 kinase activity by methylselenocysteine in synchronized mouse mammary epithelial tumor cells. *Carcinogenesis* 1997, *18*, 1541 – 1547.

[17] Weinberg, R. A., The retinoblastoma protein and cell cycle control. *Cell* 1995, *81*, 323 – 330.

[18] Bartek, J., Bartkova, J., Lukas, J., The retinoblastoma protein pathway and the restriction point. *Curr. Opin. Cell. Biol.* 1996, *8*, 805 – 814.

[19] Albino, A. P., Juan, G., Traganos, F., Reinhart, L., *et al*., Cell cycle arrest and apoptosis of Melanoma cells by docosahexaenoic acid: Association with decreased pRb phosphorylation. *Cancer Res*. 2000, *60*, 4139 – 4145.

[20] Zhu, Z., Jiang, W., Ganther, H. E., Thompson, H. J., Mechanisms of cell cycle arrest by methylseleninic acid. *Cancer Res*. 2002, *62*, 156 – 164.

[21] Nigg, E. A., Cyclin-dependent protein kinases: Key regulators of the eukaryotic cell cycle. *Bioessays* 1995, *17*, 471 – 480.

[22] Sherr, C. J., Roberts, J. M., Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* 1995, *9*, 1149 – 1163.

[23] Dong, Y., Zhang, H., Hawthorn, L., Ganther, H. E., Ip, C., Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array. *Cancer Res.* 2003, *63*, 52 – 59.

[24] Huang, R. P., Fan, Y., de Belle, I., Niemeyer, C., *et al*., Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. *Int. J. Cancer* 1997, *72*, 102 – 109.

[25] Wang, Z., Jiang, C., Ganther, H. E., Lu, J., Antimitogenic and proapoptotic activities of methylseleninic acid in vascular endothelial cells and associated effects on PI3K-AKT, ERK, JNK and p38 MAPK signaling. *Cancer Res.* 2001, *61*, 7171 – 7178.

[26] Garcia, S., Dales, J. P., Charafe-Jauffret, E., Carpentier-Meunier, S., *et al*., Overexpression of c-Met and of the transducers PI3K, FAK and JAK in breast carcinomas correlates with shorter survival and neoangiogenesis, *Int. J. Oncol.* 2007, *31*, 49 – 58.

[27] Hei, Y. J., Farahbakhshian, S., Chen, X., Battell, M. L., McNeill, J. H., Stimulation of MAP kinase and S6 kinase by vanadium and selenium in rat adipocytes. *Mol. Cell. Biochem*. 1998, *178*, 367 – 375.

