Selenite triggers rapid transcriptional activation of p53, and p53-mediated apoptosis in prostate cancer cells: Implication for the treatment of early-stage prostate cancer

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Abstract. Supra-nutritional selenium supplementation has emerged as an attractive new approach to intervene in a range of human cancers, in particular prostate cancer. However, scanty information is currently available on molecular mechanisms underlying selenium's anticancer action. The tumor suppressor p53 plays an important role in preventing transformation by transcriptional regulation of a range of genes that are involved in vital cell functions such as DNA repair, cell cycle arrest, and induction of apoptosis. Here we report that incubation of LNCaP human prostate cancer cells (*p53* +/+) with a natural form of selenium triggers rapid transcriptional activation of p53, and up-regulation of the expression of p53-target genes as well as induction of miR-34 class of microRNAs. Moreover, blocking p53 function by transfection of cells with a dominant-negative, mutated *p53* gene, or by siRNA, significantly reduced selenium-induced expression of p53-target genes and induction of apoptosis. Since majority of the early-stage human prostate cancers bear functional *p53* gene (*p53*+/+), our findings indicate that the anticancer action of selenium may involve transactivation of *p53* as a potential mechanism, and suggest that selenite may be useful not only for prevention but also for treatment of human prostate cancer.

Introduction

Use of selenium as dietary supplement has turned out to be an attractive new approach to intervene a range of human cancers, such as prostate cancer, lung cancer, colon cancer (1-3). This initiative is gathering momentum after the phenomenal discovery by Clark *et al* that showed ~63% reduction of prostate cancer incidence with nutritional supplementation of selenium as selenized yeast (4). Higher toe nail selenium levels were observed to be associated with a 50% reduced risk of metastatic prostate cancer (5). Role of selenium to reduce the risk of prostate cancer has been suggested by epidemiologic studies (6,7), and recently supported by randomized intervention trial, which indicated that a 4 to 5-fold increased risk of prostate cancer is associated with low plasma selenium (8). Supplementation of dietary selenium is now gaining support both for the prevention and treatment of cancer based on the earlier findings and recent experimental data, though the results are not always conclusive as predictors of cancer development (9), which suggests for the existence of additional factors influencing the outcome of the use of selenium in cancer management. As an essential trace element, selenium plays an important role as component of the active site of several cellular enzymes, particularly those responsible for detoxification (10,11). Thus, selenium is considered to be involved in the regulation of various cell functions including cell growth and apoptosis (12). However, precise mechanisms of selenium's action preventing development and/or progression of prostate cancer are yet to be understood.

Selenium comes from both organic and inorganic sources. Natural selenium species, such as sodium-selenite (Na_2SeO_3) and selenomethionine (SeMet) were observed to inhibit monolayer and anchorage-independent growth of a series of normal and cancerous prostate epithelial cells at various concentrations (13,14). Long-term treatment of LNCaP prostate cancer cells with selenite exhibited mitochondrial injury and cell death (15). Thus, one possible mechanism of how selenium may control prostate cancer is by reducing cell growth and inducing cell death. Selenium has recently been reported to increase the activity of stress kinase to induce apoptosis in oral carcinoma cells and is known to affect cellular redox status by generating oxidative radicals that are deleterious to cell survival (16-18). Selenide (a metabolite of selenite) is known to interfere with the action of lipoxygenases, in particular 5-lipoxygenase (5-Lox) by active site modification (19-22). Selenium increases the activity of glutathione peroxidase (GPx) including phospholipid hydroperoxide glutathione peroxidase (PHGPx) which by lowering cellular peroxide tone down-regulates the activity of cyclooxygenase

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and lipoxygenase enzymes (23). We reported earlier that selenium triggers caspase-dependent apoptosis in LNCaP human prostate cancer cells (but not in normal prostate epithelial cells), which is effectively prevented by exogenous metabolites of 5-Lox, 5(S)-HETE and 5-oxoETE (24), suggesting that direct effect of selenium on cancer cells may include inhibition of 5-Lox activity. Later on, we observed that the p53-positive LNCaP cells are more sensitive to selenite than the p53-negative PC3 prostate cancer cells, suggesting that p53 may also play a role in selenium's effect on prostate cancer cells.

The tumor suppressor p53 is known to play a vital role in preventing the development and progression of cancer by virtue of its control over a range of other genes involved in a variety of cell functions, such as DNA repair, cell cycle arrest and induction of apoptosis, and thus p53 is aptly regarded as the 'Guardian of the genome' (25-28). p53 is a multi-domain protein, and is known to be activated by post-translational modifications (such as phosphorylation and acetylation) under conditions of various cellular emergencies (DNA-damage, stress stimuli) and to react promptly both in transcription-dependent and transcription-independent manner. The N-terminal domain of p53 (residues 1-73) regulates its transactivation function and harbors at least eight phosphorylation sites: serine 6, 9, 15, 20, 33, 37, 46, and Threonine 18 (25-29). Phosphorylation of the serine-15 residue plays a critical role in the transactivation function of p53 and happens soon after initiation of stress (30,31). p53 was observed to be required for induction of apoptosis in response to oxidative stress and radiation exposure, and found to be associated with chemotherapy-induced cell death (32-34). A well characterized mechanism for p53 function is by induction of a number of target genes, such as p21, Bax, Noxa, PUMA, through transactivation (35-38). Since the apoptosis-inducing role of p53 is well characterized, we hypothesized that selenite-induced apoptosis in prostate cancer cells includes activation of p53 which also underlies as a mechanism for the differential sensitivity between p53-positive and p53-negative cancer cells.

Phosphorylation of p53 at serine-15 residue has been observed after selenium treatment (39), suggesting that this phenomenon may be linked with the transcriptional activation of p53. However, details of the transcriptional activation of p53 by selenite (nuclear localization, DNA binding, transcription of target genes), its temporal relation to DNA damage signals, and the role of p53 in the regulation of selenite-induced apoptosis in prostate cancer cells are yet to be documented. We report here that selenite triggers rapid transcriptional activation of p53 in LNCaP prostate cancer cells which is detectable within 1-2 h post treatment, and that p53 plays an important role in selenite-induced apoptosis. Since, majority of the early-stage prostate cancers bear functional *p53* gene (40), our findings suggest that the anticancer action of selenium may include transcriptional activation of p53 as a potential mechanism.

Materials and methods

Cell culture and reagents. LNCaP human prostate cancer cells were purchased from American Type Culture Collection (Manassas, VA). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) plus 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37˚C in humidified CO_2-incubator. Cells were fed with fresh medium every third day and passaged at a confluence of ~80%. Sodium selenite (Na_2SeO_3) plus other fine chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Pifithrin-α was purchased from Calbiochem (La Jolla, CA). Polyclonal antibodies against p53, phospho-p53, histone-H2A.X, phospho-H2A.X, were purchased from Cell Signaling (Danvers, MA). Alexa-fluor 488-labeled goat anti-rabbit antibody was purchased from Invitrogen. Antibodies against p21, Bax, PIG3, and DR5 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blot. For Western blot, cells (~$3x10^5$) were plated in RPMI medium 1640 supplemented with 10% FBS in 60 mm diameter dishes (Falcon) and allowed to grow for 48 h. The spent culture medium was then replaced with 2 ml fresh RPMI medium and the cells were treated with 2.5 μM selenite with or without other agents for varying periods of time. After treatment, cells were harvested, washed, and lysed in lysis buffer (50 mM HEPES buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM vanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1% NP-40, and a cocktail of protease inhibitors). After removal of cell debris, proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat-milk solution and then blotted with appropriate primary antibody followed by peroxidase-labeled secondary antibody. Bands were visualized by enhanced chemiluminescence (ECL) detection technique (Amersham Corporation).

Microscopy. LNCaP prostate cancer cells (~$2x10^5$) were plated in RPMI medium 1640 supplemented with 10% FBS on cover glasses in 6-well tissue culture plates (Falcon) and allowed to grow for 48 h. The old medium was then replaced with 2 ml fresh RPMI medium and the cells were then treated with 2.5 μM selenite for varying periods of time at 37˚C in the CO_2 incubator. At the end of incubation periods, cells were fixed in 4% formaldehyde and permeabilized with 0.25% Triton X-100. After washing, cells were treated with primary rabbit anti-human p53 antibody overnight followed by secondary anti-rabbit IgG conjugated with Alexa-Fluor-488. Cover glasses were mounted on glass slides using VectaShield (with DAPI to counterstain nuclei) from Vector Laboratories, Burlingame, CA, and observed under Nikon fluorescence microscope. Photographs were taken with a digital camera at 40X. Image acquisition and data processing were done with a Dell computer attached to the microscope using SPOT-Advanced software.

Isolation of nuclei. To detect nuclear accumulation of p53 after selenium treatment, LNCaP cells (~10^6 per plate) were plated in 100 mm diameter culture plates and allowed to grow for 48 h. Cells were then treated with 2.5 μM sodium selenite for varying periods of times. At the end of incubation periods, cells were harvested, washed and fractionated into nuclear and cytosolic components using a kit following a two-step protocol supplied by the manufacturer (Qiagen, Valencia, CA). Cytosolic (Cyt) and nuclear (Nu) proteins were processed for Western blot as described above. For purity

analysis Lamin B and GAPDH were used as markers for nucleus and cytosol, respectively.

Transcription factor-DNA binding assay. For measuring the DNA-binding activity of p53 using specific p53-binding DNA sequence, LNCaP prostate cancer cells were plated and treated with 2.5 μM selenite as described above for isolation of nuclei. Nuclear fractions were then prepared and the DNA-binding activity of nuclear p53 with its consensus DNA sequence was measured using TransAM-p53 kit using 4 μg of nuclear proteins per assay following protocol supplied by the manufacturer (Active Motif, Carlsbad, CA).

RT-PCR. For detecting transcription of p53-target genes after selenium treatment by reverse-transcriptase PCR, LNCaP prostate cancer cells (10^6 cells per plate) were plated in 100 mm diameter tissue culture plates and treated with selenium (2.5 μM selenite) for varying periods of time. After treatment, total RNAs were isolated using Qiagen mini kit (Qiagen, Valencia, CA). First strand cDNA synthesis and PCR reactions were performed using RT-Plus PCR kit (5-PRIME, Minneapolis, MN). Gene-specific primer sets were purchased from Integrated DNA Technologies (Coralville, IA) that are matched with Santa Cruz website (http://genome.ucsu.edu/). The following primer sets were used for PCR: p21 (upstream 5-TTAGCAGCGGAACAAGGAGT-3', downstream 5'-ATT CAGCATTGTGGGAGGAG-3'); Bax (upstream 5'-ATC CAGGATCGAGCAGGGCG-3', downstream 5'-GGTTCTG ATCAGTTCCGGCA-3'); DR5 (upstream 5'-AAGACCCTT GTGCTCGTTG-3', downstream 5'-TCACCTGAATCACA CCTGG-3'); GAPDH (upstream 5'-TGAAGGTCGGAGTC AACGGATTTGGGT-3', downstream 5'-CATGTGGGCCA TGAGGTCCACCAC-3'). PCR products were separated by 1.5% agarose gel and detected with ethidium bromide staining. Bands were analyzed with Eagle Eye II Darkroom Cabinet still-video imaging system using EagleSight-v3.1 software (Stratagene, La Jolla, CA).

Real-time PCR. For detecting expression of hsa-miR-34a/b/c, LNCaP cells were plated and treated with 2.5 μM selenite as described above, and total RNAs were isolated using mirVana miRNA isolation kit (Ambion catalog no. 1561). RNA concentration was measured by NanoDrop ND-1000 spectrophotometer, and reverse transcription was performed using TaqMan MicroRNA Reverse Transcription kit from Applied Biosystems (ABI no. 4366596). Stem-loop real-time PCR of hsa-miR-34a, hsa-miR-34b, hsa-miR-34c, and RNU6B (endogenous control) were performed using TaqMan MicroRNA Assay kits (ABI no. 4373278, no. 4373037, no. 4373036 and no. 4373381, respectively) on an ABI 7500 real time PCR system for 40 cycles. All PCR reactions were performed three times in quadruplicates and gene expression was normalized to RNU6B. Data analyses were performed with 7500 system SDS software (ABI). Statistical analyses were performed with Prism 4.03 software (GraphPad, Inc., San Diego, CA).

Apoptosis measurement. Apoptosis was quantitatively measured by detecting degradation of nuclear DNA to nucleosomal fragments by sandwich-ELISA. Prostate cancer cells (~300,000 per plate) were plated in 60 mm diameter tissue culture dishes (Falcon) and allowed to grow for 48 h. Cells were then treated either with the experimental agents or solvent controls for periods up to 16 h. At the end of incubation periods, cells were harvested, lysed, and the degradation of nuclear DNA to nucleosomal fragments was studied by Cell Death Detection ELISA plus following instructions supplied by the manufacturer (Roche, Indianapolis, IN). Cell lysates equivalent to 4,000 cells were used per assay.

RNA interference and gene-targeting. To understand the role of p53 in selenium-induced apoptosis, LNCaP cells ($3x10^5$) were plated in 60 mm diameter plates in duplicates. The next day, cells were treated with siRNA against p53, or control-siRNA, mixed with non-toxic doses of lipid-based transfection reagent (MIRUS, Madison, WI) for 48 h at 37˚C in the incubator. Then the cells were treated with 2.5 μM selenite, or media only, for 16 h. At the end of incubation period, one set of cells were used for Western blot analysis of p53 and its target genes (p21, Bax, DR5, PIG3), and the duplicate set of cells were used for apoptosis measurement by Cell Death ELISA.

In another set of experiments, parental LNCaP cells and the dominant-negative mutated *p53* gene-transfected LNCaP cells (LN-P151S; courtesy of Dr R. deVere White, UC Davis, CA) were plated and treated with 2.5 μM selenite for 16 h. At the end of incubation period, cells were lysed and one set of cells were used for Western blot analysis of p53 and p53-target genes, and the duplicate set was used for apoptosis measurement. Apoptosis was measured by Cell Death ELISA.

Results

Selenite triggers rapid phosphorylation of the tumor suppressor p53 at the N-terminal serine-15 residue. To understand the molecular mechanism of selenium-induced apoptosis in prostate cancer cells, we analyzed early events associated with mitochondrial and cell death receptor-mediated apoptosis pathways. Since we observed that physiological selenium readily induces apoptosis in the *p53*-positive (*p53* +/+) LNCaP prostate cancer cells, whereas the *p53*-negative (*p53*-/-) PC3 prostate cancer cells requires higher dose, we tested whether the selenium-induced apoptosis in LNCaP prostate cancer cells involves activation of the tumor suppressor p53. Phosphorylation of p53 at the N-terminal serine-15 residue is known to be linked with its transcriptional activation. We tested whether treatment with selenite phosphorylates p53 at the serine-15 residue in LNCaP prostate cancer cells. We observed that selenium (2.5 μM sodium selenite) induces phosphorylation of p53 at serine-15 residue in a time-dependent manner, which is detectable within an hour post treatment (Fig. 1). Amount of total p53 protein was also increased upon selenite treatment presumably due to stabilization of p53 protein after phosphorylation. These findings show a rapid effect of selenium on phosphorylation and stabilization of the tumor suppressor p53 prostate cancer cells.

Selenium-induced phosphorylation of p53 at serine-15 in prostate cancer cells is independent/upstream of DNA

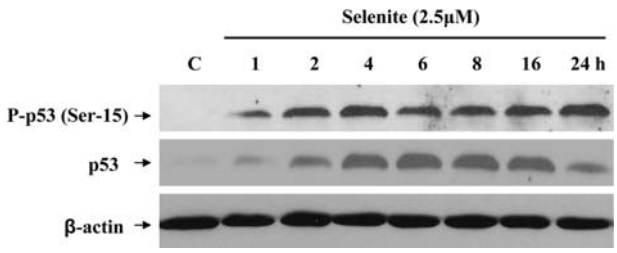


Figure 1. Phosphorylation of p53 in prostate cancer cells by selenite treatment. LNCaP prostate cancer cells (~3x10^5 per plate) were treated with selenium (2.5 μM sodium selenite) at 37˚C for time as indicated. Control cells were treated with the culture medium only. Cell lysate proteins (50 μg per lane) were resolved in SDS-PAGE and blotted with polyclonal anti-phospho-serine^{15}-p53 antibody followed by secondary HRP-conjugated anti-rabbit polyclonal antibody. Bands were visualized by ECL detection technique. The membrane was stripped and reprobed with a rabbit anti-p53 antibody to show total p53 proteins. ß-actin was used as a loading control. Results show a representative of three separate experiments with similar results.

damage signals. Phosphorylation of p53 at serine-15 is known to occur upon DNA damage signals (double strand breaks) via activation of ATM and other related kinases. Recently, DNA-damage independent activation of ATM kinase and phosphorylation of p53 at serine-15 has been observed upon treatment with chloroquine and indole-3-carbinol (41,42). Thus, we wanted to know whether DNA double strand breaks occur in LNCaP cells upon treatment with selenite. Interestingly, we observed that phosphorylation of the DNA damage-indicator histone, (H2A.X), occurs only at 4-6 h post selenium treatment (Fig. 2A). The phosphorylation of H2A.X corresponds to the apoptotic degradation of chromatin DNA to nucleosomal fragments (Fig. 2B) which involves DNA 'double strand breaks'. Thus, our results suggest that selenium-induced phosphorylation of p53 at serine-15 residue is an earlier event than phosphorylation of H2A.X at serine-139 which correlates with DNA breakage.

Selenium induces enrichment of p53 in the nuclei within hours of treatment. Phosphorylation of p53 at the N-terminal serine-15 residue is particularly significant because, i) it dissociates p53 from the E3 ubiquitin ligase HDM2 (MDM2 in mice) which promotes degradation of p53 via ubiquitination, and ii) it blocks the nuclear export signal of p53 (33,34,43,44). Thus, phosphorylation of p53 at serine-15 residue increases nuclear accumulation of p53 molecules. We wanted to investigate whether selenite-induced phosphorylation of p53 in prostate cancer cells leads to an increase in the accumulation of p53 in the nuclei. We observed that when LNCaP prostate cancer cells were treated with selenite, a pronounced accumulation of p53 occurs within the nuclei in a time-dependent manner (Fig. 3A). In control, 4 out of 38 cells (10.5%) showed increased levels of p53, whereas 11 out of 32 (34.3%), 17 out of 38 (44.7%), and 18 out of 21 (85.7%) cells showed increased levels of nuclear p53 after 1, 2 and 4 h of selenite treatment, respectively (Fig. 3B). Nuclear accumulation of both phosphorylated and total p53 proteins were also observed to be increased by Western blot analysis upon selenium treatment (Fig. 3C).

Increased DNA-binding activity of nuclear p53 in prostate cancer cells upon selenium treatment. Increased nuclear accumulation of p53 upon selenium treatment was suggestive of transcriptional activation of p53 which involves tetramerization and DNA-binding. We next examined the DNA-binding activity of nuclear p53 using consensus sequence of p53-binding DNA element. The p53 consensus sequence was initially defined as two copies of the 10-bp motif consensus DNA sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs (45). We used the following DNA sequence, 5'-GGACATGCCCGGGCATGTCC-3', for the p53 binding assay. We observed that the binding activity of nuclear p53 with its consensus sequence of DNA increased in a time-dependent manner which is detectable as early as an hour post treatment (Fig. 4). Moreover, the DNA-binding

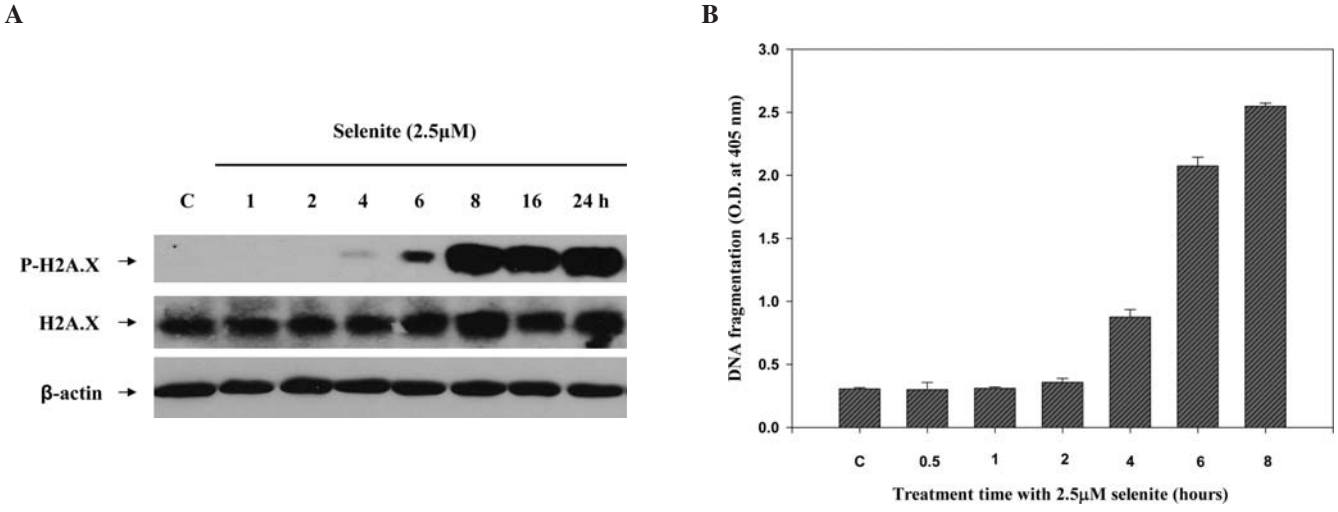
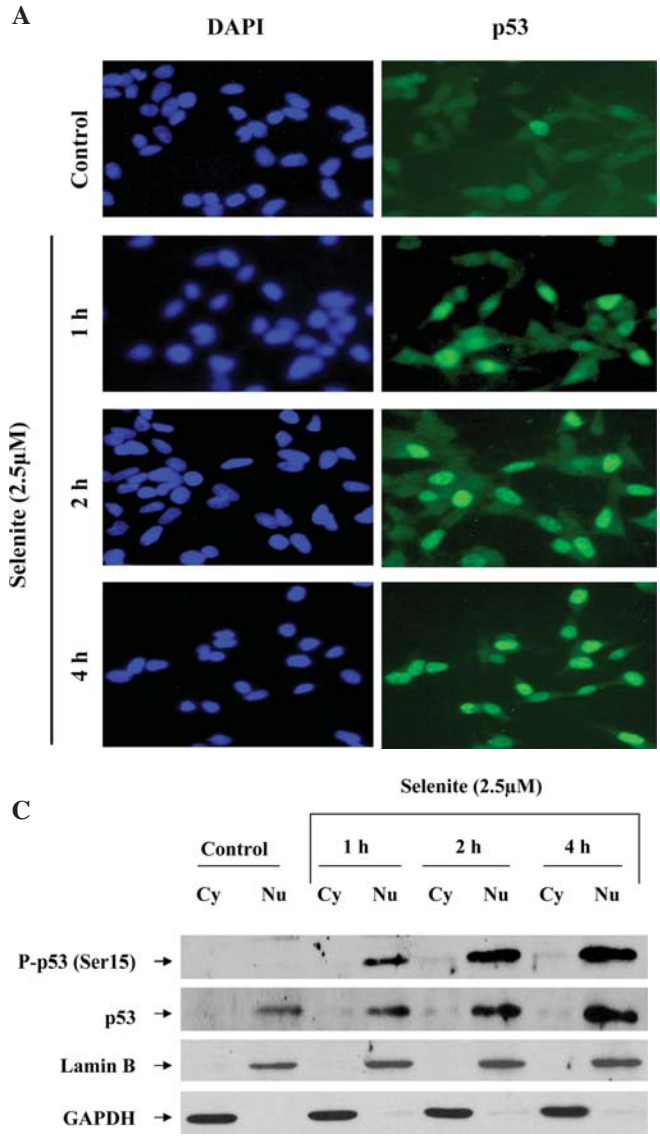


Figure 2. (A) Phosphorylation of histone H2A.X by selenium treatment in prostate cancer cells. LNCaP cells (~3x10^5 per plate) were plated, treated with selenite (2.5 μM), and proteins were separated as in Fig. 1 above. The nitrocellulose membrane was probed with phospho-serine^{139}-H2A.X antibody and secondary HRP-conjugated anti-rabbit polyclonal antibody. The same membrane was stripped and reprobed with a general H2A.X antibody to show total H2A.X proteins. Bands were visualized by ECL detection technique. (B) Induction of apoptosis in prostate cancer cells by selenium. LNCaP cells (~3x10^5) were plated overnight and allowed to grow for 48 h. Cells were then treated with 2.5 μM sodium selenite and incubated at 37˚C for 6 h. At the end of incubation period, apoptosis was quantitatively measured by sandwich-ELISA. Results represent mean values of each data point ± standard error (n=4).



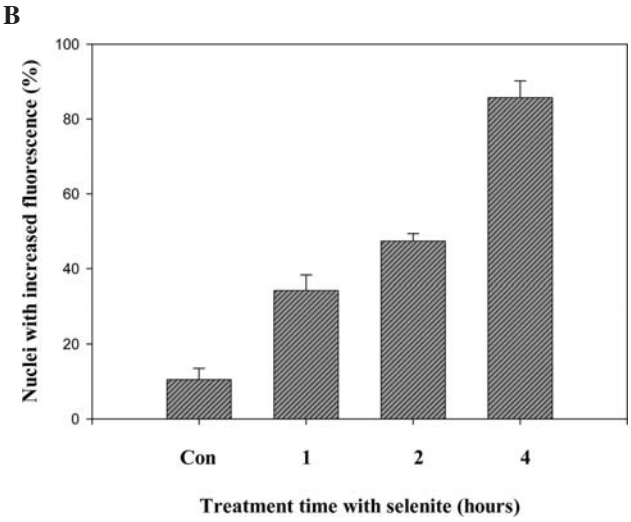


Figure 3. Nuclear accumulation of p53 after selenium treatment. (A) LNCaP cells were plated and treated with 2.5 μM sodium selenite for times as indicated. p53 was detected by treating cells with rabbit anti-p53 antibody and goat anti-rabbit secondary antibody labeled with Alex-fluor 488 (green). Nuclei were stained with DAPI (blue). (B) Graphical representation of the percentage of nuclei showing increased accumulation of p53 in the representative optical field. (C) Western blot detection of nuclear p53 after selenite treatment. LNCaP cells were treated with 2.5 μM sodium selenite for times as indicated. At the end of incubation periods, cells were fractionated into nuclear (Nu) and cytosolic (Cyt) components. Proteins (50 μg per lane) were resolved in SDS-PAGE, and detected by Western blot using anti-phosphoserine-15 or anti-p53 primary antibodies. Bands were visualized by ECL. Note: Lamin B and GAPDH were used as markers for nucleus and cytosol respectively. Results show a representative of three similar experiments.

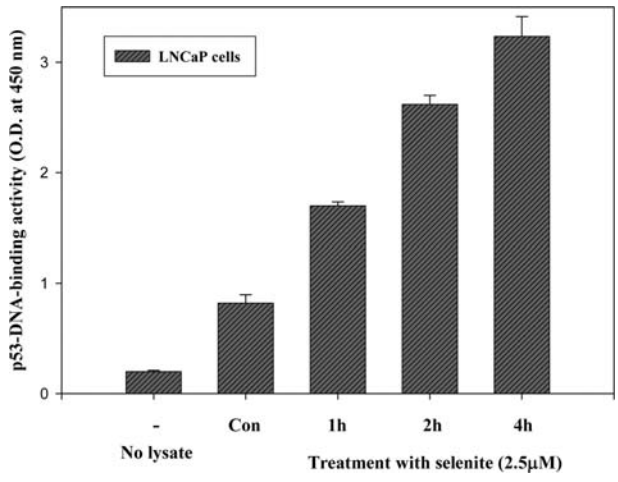


Figure 4. DNA-binding activity of p53 after selenium treatment. LNCaP cells were plated, treated with 2.5 μM selenite, and nuclear fractions were prepared as described in Fig. 3 above. Binding of nuclear p53 with its consensus DNA sequence was measured with TransAM-p53 kit using 4 μg of nuclear proteins per assay following protocol supplied by the manufacturer. Data represent mean value of triplicate determinations ± SEM.

activity of p53 positively correlated with the time course of its nuclear enrichment, suggesting that the nuclear p53 is transcriptionally active.

Treatment with selenium induces rapid transcription of p53-target genes in prostate cancer cells. To confirm that the increased DNA-binding activity of nuclear p53 in prostate cancer cells is because of transcriptional activation of p53, we examined endogenous transcription of p53-target genes upon selenite treatment in these cells. Our results show a distinct time-dependent induction of several p53 target genes (p21, Bax, and DR5) in LNCaP prostate cancer cells after selenite treatment (Fig. 5A). Interestingly, we observed that not all the p53 target genes showed uniform time-dependent induction upon selenium treatment, suggesting a preferential activation of the promoters of some genes by p53 in these cells for transcription.

MicroRNA-34 (miR-34) has recently been characterized as transcriptional target of p53 (46-48). We wanted to see whether activation of p53 by selenite also induces transcription of miR-34 in prostate cancer cells. By stem-loop RT-PCR we observed that treatment with selenite induced expression of miR-34b and miR-34c, but not miR-34a in LNCaP cells (Fig. 5B). This finding suggests that selenite-induced growth arrest and apoptosis include a microRNA component that may also play an active role in the anti-cancer action of selenium.

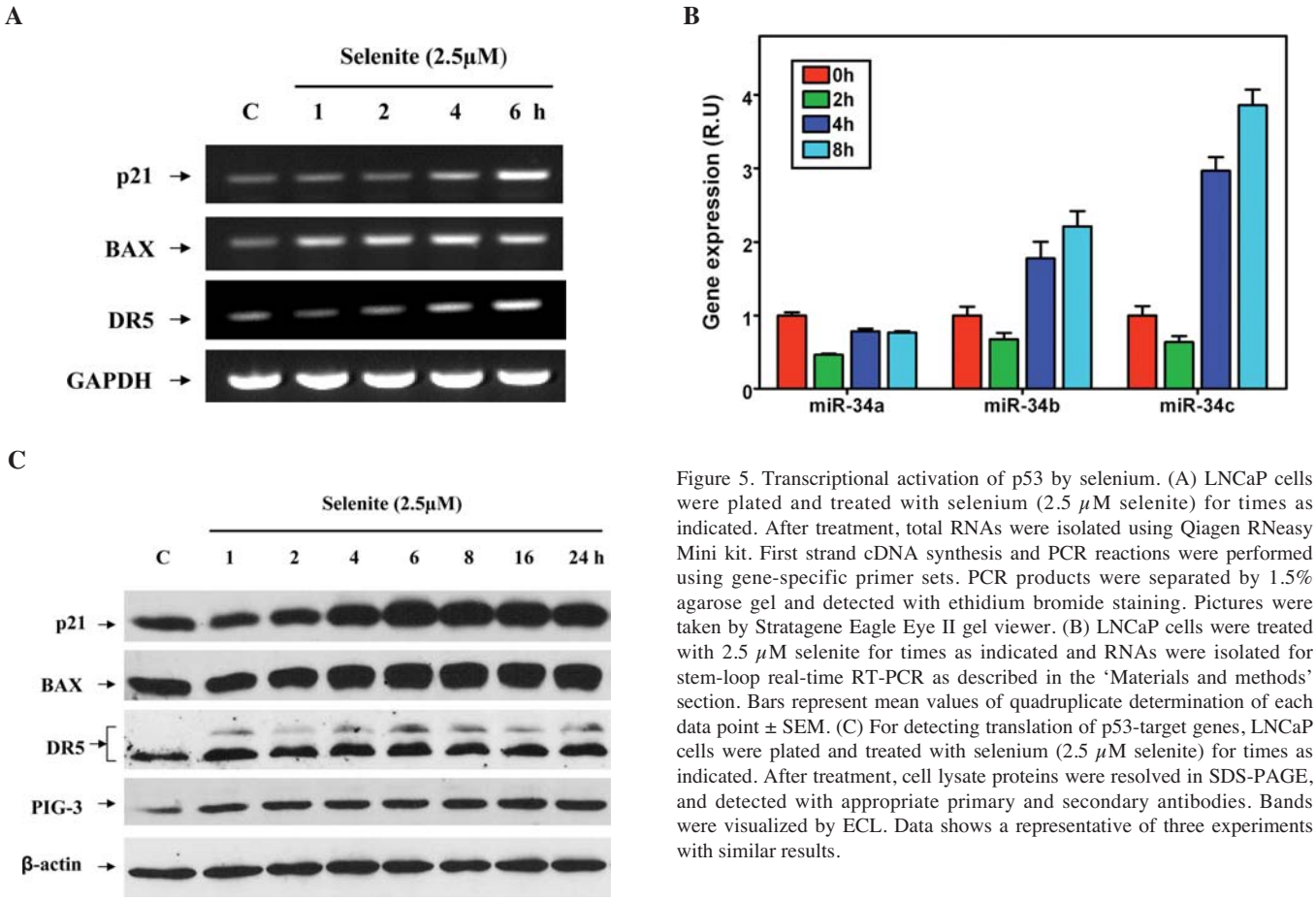


Figure 5. Transcriptional activation of p53 by selenium. (A) LNCaP cells were plated and treated with selenium (2.5 μM selenite) for times as indicated. After treatment, total RNAs were isolated using Qiagen RNeasy Mini kit. First strand cDNA synthesis and PCR reactions were performed using gene-specific primer sets. PCR products were separated by 1.5% agarose gel and detected with ethidium bromide staining. Pictures were taken by Stratagene Eagle Eye II gel viewer. (B) LNCaP cells were treated with 2.5 μM selenite for times as indicated and RNAs were isolated for stem-loop real-time RT-PCR as described in the 'Materials and methods' section. Bars represent mean values of quadruplicate determination of each data point ± SEM. (C) For detecting translation of p53-target genes, LNCaP cells were plated and treated with selenium (2.5 μM selenite) for times as indicated. After treatment, cell lysate proteins were resolved in SDS-PAGE, and detected with appropriate primary and secondary antibodies. Bands were visualized by ECL. Data shows a representative of three experiments with similar results.

We investigated whether increased production of messages of p53-target genes upon selenium treatment proceeds up to increased protein levels via translation. We observed that selenium treatment increased the protein-levels of several p53 target genes, such as p21, Bax, DR5, and PIG-3 (Fig. 5C). Interestingly, increase in protein levels of p53 target genes was observed at different time points after selenium treatment with p21 being the earliest.

The tumor suppressor p53 plays a causal role in selenite-induced apoptosis in prostate cancer cells. Rapid activation of p53 via phosphorylation upon selenite treatment prompted us to verify whether the tumor suppressor p53 plays any role in selenium-induced apoptosis induction in prostate cancer cells. We addressed this question by inhibiting p53 function in LNCaP cells by pre-treatment with a pharmacological inhibitor of p53, pifithrin α. We observed that selenium-induced apoptosis in LNCaP cells was significantly prevented when the cells were pre-treated with pifithrin-α, suggesting that activation of p53 in prostate cancer cells may play a causal role in selenium-induced apoptosis (Fig. 6A).

In another set of experiments, we used a derivative of LNCaP prostate cancer cells stably transfected with a dominant-negative mutated *p53* gene (LNCaP-P151S) and treated these cells with selenium in parallel experiment with parental LNCaP cells in the same experimental conditions. We observed marked reduction of apoptotic DNA degradation in LNCaP-P151S cells by selenium which is also accompanied by dramatic down-regulation of the expression of p53 target genes, such as p21, Bax, and PIG-3 (Fig. 6B).

We also tested the effect of p53 knockdown on selenium-induced apoptosis in LNCaP prostate cancer cells by treating cells with small interfering-RNA (siRNA) against p53. Our results show that treatment of cells with siRNA against p53 reduced the expression of p53 as well as p53-target genes, and also reduced selenium-induced apoptotic degradation of DNA (Fig. 6C). These observations suggest that the tumor suppressor p53 plays an active role in selenium-induced apoptosis in prostate cancer cells.

Discussion

We observed that selenite triggers rapid phosphorylation of the tumor suppressor p53, at the N-terminal Box-1 serine-15 residue which is apparent as early as 60 min post treatment (Fig. 1). We also observed increased levels of p53 protein upon selenium treatment, suggesting for its stabilization presumably due to phosphorylation at serine-15. The p53 tumor suppressor gene produces a highly labile protein transcription factor that is activated by a wide range of cellular stresses. Phosphorylation of serine-15 prevents binding of p53 with MDM2 which otherwise degrades p53 through ubiquitination and proteasomal action (43,44), and helps p53 for its transcriptional activation by increasing the level of nuclear p53. Additionally, Ser-15 phosphorylation increases p53-dependent transcription via modulating its affinity to interact with transcriptional co-activators such as p300/CBP (49). Thus, phosphorylation of p53 protein at the serine-15 residue is an indicator of its transcriptional activation.

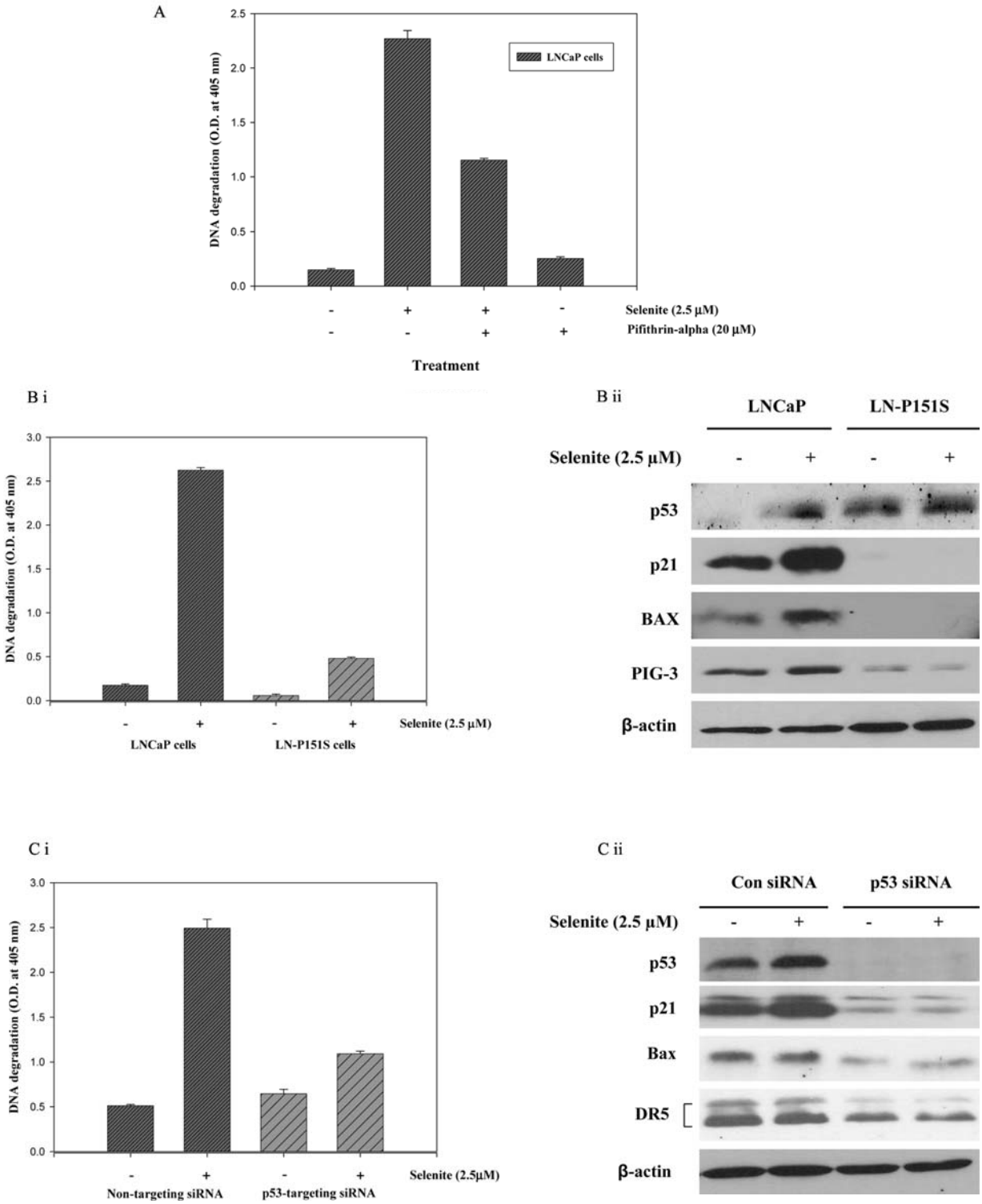


Figure 6. Role of p53 in selenium-induced apoptosis. (A) LNCaP cells were plated as in Fig. 5 and pre-treated with 20 μM pifithrin-α for 24 h. Then the cells were treated with 2.5 μM selenite for 16 h. At the end of incubation period, apoptosis was measured by ELISA. (B) Control LNCaP cells, and the dominant-negative mutated *p53* gene-transfected LNCaP cells (LN-P151S) were plated and treated with 2.5 μM selenite for 16 h. (i) Apoptosis was measured by ELISA. Right panel (ii), shows expression of p21, Bax and PIG3 proteins in LNCaP and LN-P151S cells with and without selenium treatment. Note: Decrease in p53 function in LN-P151S cells (as apparent by expression of p53-target gene products), positively correlates with decreased selenium-induced apoptosis. (C) LNCaP cells were treated with control non-targeting siRNA or p53-siRNA for 48 h. Then the cells were treated with 2.5 μM selenite for 16 h. Apoptosis and expression of proteins were measured as described in (B).

The p53 protein is phosphorylated by a range of kinases that are activated under a variety of stress conditions, and both DNA damage-dependent and DNA damage-independent activation of p53 are now known (41-43). Phosphorylation of p53 at serine-15 is known to occur via activation of ATM and ATR kinases upon DNA damage. However, DNA damage-independent phosphorylation of serine-15 has been reported (41,42), and a range of kinases other than ATM and

ATR have also been observed to phosphorylate p53 at the N-terminal serine-15 residue (50-52). We wanted to explore whether selenite-induced p53 serine-15 phosphorylation takes place when detectable DNA damage signals occur, because both for (39) and against (53) this notion have been reported previously. We observed that phosphorylation of the DNA damage-indicator histone (H2A.X) occurs only at 4 h post selenium treatment or later, which suggests that the early phosphorylation of p53 at the N-terminal serine-15 residue (detectable at 1-2 h post treatment) is an upstream event and thus this phosphorylation of p53 may not be dependent on DNA damage signals which induces phosphorylation of H2A.X (Fig. 2). Recently, activation of ATM kinase by chromatin modification (but without involving DNA damage) has been observed (54), which opens up the possibility that selenite-induced early phosphorylation of p53 may be mediated by chromatin modification rather than DNA degradation, or via some other mechanisms. Further study is needed to better understand this.

We observed an abrupt increase in the total amount of p53 accumulation in the nuclei after selenite treatment, suggesting for transcriptional activation of p53 molecules (Fig. 3). Interestingly, Western blot experiments revealed that majority of the phosphorylated (at serine-15 residue) p53 species were associated with the nuclear fraction (Fig. 3C). Nuclear accumulation of serine-15 phosphorylated-p53 is consistent with the literature which correlates with interference of the nuclear export signal of p53 molecule. A small fraction of residual p53 was also found in the nuclear fraction without detectable phosphorylation at serine-15 residue. The p53 proteins without serine-15 phosphorylation may also represent functional p53 species, as it was reported that serine-15 phosphorylation is dispensable for p53 transcriptional activity (55). The p53-DNA-binding activity observed in the untreated control sample coincided with the residual (not phosphorylated at serine-15) amount of p53 (Fig. 4). We also observed that the amount of nuclear p53 is proportional with its binding to the consensus DNA sequence, suggesting that the nuclear p53 is transcriptionally active.

Activated p53 molecules bind with promoters of target genes having functional p53-binding elements to turn on expression of those genes. Our observation of the increased production of mRNAs of p21, Bax, and DR5, and micro-RNAs (miR-34b miR-34c) suggest that treatment of prostate cancer cells with selenite triggers transcriptional activation of p53 (Fig. 5A and B). We did not observe induction of miR-34a in the same experimental condition. MiR-34a is located in chromosome 1 at the p36 locus (Chr1p36) which is frequently deleted in many cancer types (48). Deletion of miR-34a gene or inactivation of miR-34a promoter sequence may be underlying reasons for its non-induction in LNCaP cells which is not known at this time. Alternatively, these observations may indicate differential regulation of miR-34a and miR-34b/c in response to selenite treatment. We also observed that treatment of LNCaP cells with selenium increases protein products of p53-target genes suggesting that the activation of p53 proceeds up to translation of the transcripts of p53-target genes (Fig. 5C).

Both p53-dependent and p53-independent apoptosis processes are known to occur in cells due to various toxic insults. Rapid transcriptional activation of p53 in prostate cancer cells by selenium prompted us to explore whether the tumor suppressor p53 plays any role in selenium-induced apoptosis process in these cancer cells. Our functional knock-out studies with siRNA against p53, as well as transfection of LNCaP prostate cancer cells with dominant-negative mutated p53 gene (*p53-P151S*), showed that the tumor suppressor p53 plays a causal role in selenium-induced apoptosis in these cancer cells (Fig. 6). Moreover, we observed that this concentration of selenium (2.5 μM) does not trigger detectable apoptosis in the *p53*-negative (*p53*-/-) PC3 human prostate cancer cells (not shown), which suggests that physiologically achievable doses of selenium may not show the same effect in cells where p53 function is compromised due to mutation or deletion of the p53 gene.

Choice of appropriate seleno-compounds and *in vivo* monitoring of selenium's effects will be possible only if we have proper molecular understanding about how selenium exerts its anticancer action. Our present findings show that selenite (a natural form of selenium) at physiologically achievable doses can transcriptionally activate the tumor suppressor p53 in prostate cancer cells, and that the selenite-induced apoptosis in prostate cancer cells is dependent on the function of p53. Selenium was observed to show significant effect in reducing incidence of prostate cancer, but no noticeable benefit was perceived in preventing recurrence of skin cancer (4). Though the actual reason for this discrepancy is unknown, occurrence of high rate of p53 mutation in skin cancer may be one plausible contributing mechanism, which needs further testing. Selenomethionine was selected for testing in the SELECT (Selenium and Vitamin E Cancer Prevention Trial) clinical trial for its efficacy in prostate cancer prevention which showed no beneficial effect (56). The NPC trial used selenized yeast (cultured in selenite solution) as the source of selenium (4) which included both inorganic and organic species. We observed that while selenite effectively induces apoptosis in prostate cancer cells at physiologically achievable concentrations (2-3 μM), selenomethionine at even higher concentrations (50-100 μM) exerts no detectable effect on apoptosis (not shown). Unexpected results in the SELECT trial suggest that seleno-methionine may not be the right form of selenium for prostate cancer prevention, and extensive *in vivo* testing of various selenium species is needed to resolve this issue. Our findings suggest that selenite may be an appropriate form of selenium not only for prostate cancer prevention but also for treatment of prostate cancer, and that selenium's anticancer action may be substantially compromised where the function of p53 is deregulated by mutation or deletion of the p53 gene, or by endogenous and exogenous factors that interfere with the activity of p53 molecule.

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References

1. Greenwald P, Milner JA, Anderson DE and McDonald SS: Micronutrients in cancer chemoprevention. Cancer Metastasis Rev 21: 217-230, 2002.
2. Pathak SK, Sharma RA and Mellon JK: Chemoprevention of prostate cancer by diet-derived antioxidant agents and hormonal manipulation (Review). Int J Oncol 22: 5-13, 2003.
3. Combs GF: Impact of selenium and cancer-prevention findings on the nutrition-health paradigm. Nutr Cancer 40: 6-11, 2001.
4. Clarck LC, Combs GF, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross G, Krongrad A, Lesher JL, Park HK, Sanders BB, Smith CL and Taylor JR: Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin: a randomized controlled trial. JAMA 276: 1957-1963, 1996.
5. Yoshizawa K, Willet WC, Morris SJ, Stampfer MJ, Spiegelman D, Rimm EB and Giovannucci E: A study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. J Natl Cancer Inst 90: 1219-1224, 1998.
6. Helzlsouer KJ, Huang HY, Alberg AJ, Hoffman S, Burke A, Norkus EP, Morris JS and Comstock GW: Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer. J Natl Cancer Inst 92: 2018-2023, 2000.
7. Nomura AM, Lee J, Stemmermann GN and Combs GF: Serum selenium and subsequent risk of prostate cancer. Cancer Epidemiol Biomarkers Prev 9: 883-887, 2000.
8. Brooks JD, Metter EJ, Chan DW, Sokol LJ, Landis P, Nelson WG, Muller D, Andres R and Carter HB: Plasma selenium level before diagnosis and the risk of prostate cancer development. J Urol 166: 2034-2038, 2001.
9. Goodman GE, Schaffer S, Bankson DD, Highes MP and Omenn GS: Carotene and retinol efficacy trial co-investigators, predictors of serum selenium in cigarette smokers and the lack of association with lung and prostate cancer risk. Cancer Epidemiol Biomarkers Prev 10: 1069-1076, 2001.
10. Burk RF: Selenium, an antioxidant nutrient. Nutr Clin Care 5: 75-79, 2002.
11. Brigelius-Flohe R: Tissue-specific functions of individual glutathione peroxidases. Free Radical Biol Med 27: 951-965, 1999.
12. Kim YS and Milner J: Molecular targets for selenium in cancer prevention. Nutr Cancer 40: 50-54, 2001.
13. Redman C, Scott JA, Baines AT, Basye JL, Clark LC, Calley C, Roe D, Payne CM and Nelson MA: Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. Cancer Lett 125: 103-110, 1998.
14. Menter DG, Sabichi AL and Lippman SM: Selenium effects on prostate cell growth. Cancer Epidemiol Biomarkers Prev 9: 1171-1182, 2000.
15. Zhong W and Oberley TD: Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line. Cancer Res 61: 7071-7078, 2001.
16. Ghose A, Fleming J, El-Bayoumy K and Harrison PR: Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds: involvement of mitogen-activated protein kinase and Fas pathways. Cancer Res 61: 7479-7487, 2001.
17. Powis G, Gasdaska JR, Gasdaska PY, Berggren M, Kirkpatrick DL, Engman L, Cotgreave IA, Angulo M and Baker A: Selenium and the thioredoxin redox system: effects on cell growth and death. Oncol Res 9: 303-312, 1997.
18. Shen H, Yang C, Liu J and Ong CN: Dual role of glutathione in selenite-induced oxidative stress and apoptosis in human hepatoma cells. Free Radical Biol Med 28: 1115-1124, 2000.
19. Weitzel F and Wendel A: Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. J Biol Chem 268: 6288-6292, 1993.
20. Werz O and Steinhilber D: Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells: The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. Eur J Biochemistry 242: 90-97, 1996.
21. Straif D, Werz O, Kellner R, Bahr U and Steinhilber D: Glutathione peroxidase-1 but not -4 is involved in the regulation of cellular 5-lipoxygenase activity in monocytic cells. Biochem J 349: 455-461, 2000.
22. Hammarberg T, Kuprin S, Radmark O and Holmgren A: EPR investigation of the active site of recombinant human 5-lipoxygenase: inhibition by selenide. Biochemistry 40: 6371-6378, 2001.
23. Bjornstedt M, Odlander B, Kuprin S, Claesson HE and Holmgren A: Selenite incubated with NADPH and mammalian thioredoxin reductase yields selenide, which inhibits lipoxygenase and changes the electron spin resonance of the active site iron. Biochemistry 35: 8511-8516, 1996.
24. Ghosh J: Rapid induction of apoptosis in prostate cancer cells by selenium: reversal by metabolites of arachidonate 5-lipoxygenase. Biochem Biophys Res Commun 315: 624-635, 2004.
25. Vogelstein B and Levine AJ: Surfing the p53 network. Nature 408: 307-310, 2000.
26. Ma L, Wagner J, Rice JJ, Hu W, Levine AJ and Stolovitzky GA: A plausible model for the digital response of p53 to DNA damage. Proc Natl Acad Sci USA 102: 14266-14271, 2005.
27. Harris SL and Levine AJ: The p53 pathway: positive and negative feedback loops. Oncogene 24: 2899-2908, 2005.
28. Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 88: 323-331, 1997.
29. Prives C and Hall PA: The p53 pathway. J Pathol 187: 112-126, 1999.
30. Bean LJ and Stark GR: Phosphorylation of serines 15 and 37 is necessary for efficient accumulation of p53 following irradiation with UV. Oncogene 20: 1076-1084, 2001.
31. She QB, Chen N and Dong Z: ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. J Biol Chem 275: 20444-20449, 2000.
32. Vazquez A, Bond EE, Levine AJ and Bond GL: The genetics of the p53 pathway, apoptosis and cancer therapy. Nature Reviews. Drug Discovery 7: 979-987, 2008.
33. Yin Y, Terauchi T, Solomon GG, Aizawa S, Rangarajan PN, Yazaki Y, Kadowaki T and Barrett JC: Involvement of p85 in p53-dependent apoptotic response to oxidative stress. Nature 391: 707-710, 1998.
34. Lowe SW, Ruley HE, Jacks T and Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.
35. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T and Hannon GJ: Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 377: 552-557, 1995.
36. Miyashita T and Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293-299, 1995.
37. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N: Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288: 1053-1058, 2000.
38. Yu J, Zhang L, Hwang PM, Kinzler KW and Vogelstein B: PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 7: 673-682, 2001.
39. Jiang C, Hu H, Malewicz B, Wang Z and Lu J: Selenite-induced p53 Ser-15 phosphorylation and caspase-mediated apoptosis in LNCaP human prostate cancer cells. Mol Cancer Ther 3: 877-884, 2004.
40. Voeller HJ, Sugars LY, Pretlow T and Gelmann EP: p53 oncogene mutations in human prostate cancer specimens. J Urol 151: 492-495, 1994.
41. Brew CT, Aronchik I, Hsu JC, Sheen J, Dickson RB, Bjeldanes LF and Firestone GL: Indole-3-carbinol activates the ATM signaling pathway independent of DNA damage to stabilize p53 and induce G1 arrest of human mammary epithelial cells. Int J Cancer 118: 857-868, 2006.
42. Loehberg CR, Thompson T, Kastan MB, Maclean KH, Edwards DG, Kittrell FS, Medina D, Conneely OM and O'Malley BW: Ataxia telangiectasia-mutated and p53 are potential mediators of chloroquine-induced resistance to mammary carcinogenesis. Cancer Res 67: 12026-12033, 2007.
43. Shieh SY, Ikeda M, Taya Y and Prives C: DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 91: 325-334, 1997.
44. Prives C: Signaling to p53: breaking the MDM2-p53 circuit. Cell 95: 5-8, 1998.
45. El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1: 45-49, 1992.
46. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W and Nikitin AY: MicroRNA-34b and microRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res 67: 8433-8438, 2007.
47. He X, He L and Hannon GJ: The guardian's little helper: microRNAs in the p53 tumor suppressor network. Cancer Res 67: 1099-11101, 2007.

48. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A and Mendell JT: Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell 26: 1-8, 2007.
49. Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R and Brady JN: Phosphorylation of p53 serine-15 increases interaction with CBP/p300. J Biol Chem 273: 33048-33053, 1998.
50. Hsu YL, Cho CY, Kuo PL, Huang YT and Lin CC: Plumbagin (5-Hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. J Pharmacol Exp Ther 318: 484-494, 2006.
51. Shi R, Huang Q, Zhu X, Ong YB, Zhao B, Lu J, Ong CN and Shen HM: Luteolin sensitizes the anticancer effect of cisplatin via c-Jun NH2-terminal kinase-mediated p53 phosphorylation and stabilization. Mol Cancer Ther 6: 1338-1347, 2007.
52. Persons DL, Yazlovitskaya EM and Pelling JC: Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. J Biol Chem 275: 35778-35785, 2000.
53. Zhao R, Xiang N, Domann FE and Zhong W: Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells. Cancer Res 66: 2296-2304, 2006.
54. Kim YC, Gerlitz G, Furusawa T, Catez F, Nussenzweig A, Oh KS, Kraemer KH, Shiloh Y and Bustin M: Activation of ATM depends on chromatin interactions occurring before induction of DNA damage. Nat Cell Biol 11: 92-96, 2009.
55. Thompson T, Tovar C, Yang H, Carvajal D, Vu BT, Xu Q, Wahl GM, Heimbrook DC and Vassilev LT: Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis. J Biol Chem 279: 53015-53022, 2004.
56. Lippman SM, Klein EA, Goodman PJ, *et al*: Effect of selenium and vitamin E on risk of prostate cancer and other cancers. JAMA 301: 39-51, 2009.