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The selenium analog of the chemopreventive compound S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiurea is a remarkable inducer of apoptosis and inhibitor of cell growth in human non-small cell lung cancer

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Abstract

Lung cancer continues to be the leading cause of cancer deaths throughout the world and conventional therapy remains largely unsuccessful. Although, chemoprevention is a plausible alternative approach to curb the lung cancer epidemic, clinically there are no effective chemopreventive agents. Thus, development of novel compounds that can target cellular and molecular pathways involved in the multistep carcinogenesis process is urgently needed. Previous studies have suggested that substitution of sulfur by selenium in established cancer chemopreventive agents may result in more effective analogs. Thus in the present study we selected the chemopreventive agent S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiurea (PBIT), also known to inhibit inducible nitric oxide synthase (iNOS), synthesized its selenium analog (Se-PBIT) and compared both compounds in preclinical model systems using non-small cell lung cancer (NSCLC) cell lines (NCI-H460 and A549); NSCLC is the most common histologic type of all lung cancer cases. Se-PBIT was found to be superior to PBIT as an inducer of apoptosis and inhibitor of cell growth. Se-PBIT arrested cell cycles at G1 and G2–M stage in both A549 and H460 cell lines. Although both compounds are weakly but equally effective inhibitors of iNOS protein expression and activity, only Se-PBIT significantly enhanced the levels of p53, p38, p27 and p21 protein expression, reduced levels of phospholipase A2 (PLA2) but had no effect on cyclooxygenase-2 (COX-2) protein levels; such molecular targets are involved in cell growth inhibition, induction of apoptosis and cell cycle regulation. The results indicate that Se-PBIT altered molecular targets that are involved in the development of human lung cancer. Although, the mechanisms that can fully account for these effects remain to be determined, the results are encouraging to further evaluate the chemopreventive efficacy of Se-PBIT against the development of NSCLC in a well-defined animal model.

Keywords

Apoptosis; Lung cancer; Selenium

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

Lung cancer continues to be the leading cause of cancer deaths throughout the world. In the USA estimated new cases in 2008 is more than 215 thousands and estimated death from lung and bronchus in 2008 is 162 thousands. Tobacco smoking is the major cause of this epidemic and approximately 90 million current and former smokers in the United States have a permanently increased relative risk of developing lung cancer from which 10–18% will ultimately develop lung cancer [1,2]. Unfortunately, the available strategies including conventional therapy to prevent the lung cancer epidemic remain largely unsuccessful. For this alarming number of high-risk population, chemoprevention is a plausible alternative approach to curb the lung cancer epidemic. However, clinically there are no effective chemopreventive agents targeting lung cancer [3–6].

We and others have clearly shown that both the dose and the form of selenium are critical factors that determine the chemopreventive efficacy of selenium-based compounds. Thus, our laboratory took the initiative to develop novel forms of selenium compounds that can specifically target important mediators and pathways involved in the multistep carcinogenesis process particularly the promotion/progression phases of lung carcinogenesis, i.e. agents that can be used in ex-smokers to reduce the lingering lung cancer risk that remains high for 5–10 years after cessation of the habit.

Several studies in animal models have provided direct evidence for the role of iNOS inhibitors as chemopreventive agents; *S,S'*-(1,4-phenylenebis[1,2-ethanediyl])bis(isothioureia) (PBIT) is a representative example of this class [7–10]. Potential approaches to target iNOS, and thus NO production, have led to the development of specific iNOS inhibitors that are ι -arginine-based substrate analogs that bind directly to the iNOS active site, thereby decreasing NO production and preventing tumor development. PBIT has a structural similarity to guanidine and it competitively binds in the guanidine portion of the ι -arginine active site of iNOS, hence acts as a relatively specific iNOS inhibitor. In addition, the inhibition of the iNOS may be due, in part, to binding of the S-atom of PBIT and related isothioureia and bis-isothioureia to the active site of the enzyme [11].

Literature data suggest that sulfur substitution by selenium of established cancer chemopreventive agents resulted in more effective chemopreventive analogs (reviewed in [12]). Therefore in the present investigation we synthesized and compared the effects of PBIT and Se-PBIT in preclinical model systems using non-small cell lung cancer (NSCLC) cell lines (NCI-H460 and A549) at several doses including physiological levels (human blood levels range between 2.5 and 5 μ M in people supplemented with selenium enriched yeast at 200–400 μ g/day) on cell growth, apoptosis, cell cycle analysis and on certain molecular markers including iNOS protein expression that are known to be involved in the development of lung cancer [13].

2. Materials and methods

2.1. Synthesis of Se-PBIT and PBIT

Dihydrobromide salt of *S,S'*-1,4-phenylenebis(1,2-ethandiyl)-bis(isothioureia) (PBIT) was prepared according to previously described procedure with minor modifications as shown in Fig. 1 [11]. The key intermediate in this synthesis is 1,4-[phenylenebis(1,2-ethanediyl)dibromide] (**3**). The methyl ester of 1,4-phenylenediacetic acid (**1**) on reduction with lithium aluminium hydride gave diol, **2**. Compound **2** on bromination with carbon tetrabromide and triphenylphosphine gave the dibromo compound **3** in moderate yield. This dibromo compound **3** was reacted with thioureia to give PBIT in quantitative yield. Dihydrobromide salt of *Se,Se'*-1,4-phenylenebis(1,2-ethandiyl)bis(isoselenoureia) (Se-PBIT)

was prepared in quantitative yield by reacting dibromo compound **3** with selenourea and spectral and chromatographic characteristics of both compounds are in agreement with those reported in literature [14].

2.2. Treatment of NSCLC and normal lung cells with PBIT and Se-PBIT

Human NSCLC cell lines (NCI-H460 and A549) and normal human lung fibroblast cells obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 and EMEM respectively, containing 10% FBS in a humidified environment at 37 °C with 5% CO₂. Stock solutions of PBIT and Se-PBIT were prepared in H₂O. The cells were treated with various concentrations of PBIT and Se-PBIT in the cell culture medium for 24 h. For iNOS expression cells were pretreated with cytomix (10 ng/ml IL-1 β , 10 ng/ml TNF- α and 100 ng/ml IFN- γ) that was procured from Sigma–Aldrich, Saint Louis, MO, and starved of FBS for 24 h. Control cultures were treated with H₂O and were processed similarly.

2.3. Quantification of nitric oxide in NSCLC cells

Cells (10,000/well) were grown in a 96-well plate for 24 h and treated with respective agents for 24 h. Total nitrate was converted to nitrite by NADH-dependent nitrate reductase enzyme and total nitrite was measured spectrophotometrically using the Griess Reagent according to the manufacturer's protocol [15]. Sample measurements were based on a standard linear curve that had been constructed by using various concentrations of standard nitrate, supplied in the kit by the manufacturer [15]. Each treatment was performed in triplicate and the mean was plotted as bar diagram.

2.4. MTT assay

NCI-H460, A549 and normal human lung fibroblast cells (10,000/well) were grown in a 96-well plate for 24 h and then treated with different doses (1.25, 2.5 and 5.0 μ M) of PBIT and Se-PBIT for 24 h followed by the MTT (methylthiazolotetrazolium) treatment (50 μ g/100 μ l) to the cells in each well for 4 h at 37 °C as described earlier [16]. MTT was aspirated and 100 μ l of DMSO was added to each well and absorbance at 570 nm was read in a plate reader. Each treatment was done in triplicate. Mean of three values was determined and the results are expressed as percent of control.

2.5. Apoptosis assay

H460 and A549 cells (50,000/well) were grown for 24 h in a 24-well plate and then treated with varying doses (1.25, 2.5 and 5.0 μ M) of PBIT and Se-PBIT for 24 h. The assay was carried out using a cell death detection ELISA kit from Roche Diagnostics Corporation, Indianapolis, IN, and absorbance was read at 410 nm in a plate reader. The assay was performed in triplicate and the results are presented as enrichment factor (ratio of treatment over control).

2.6. Cell cycle analysis

H460 and A549 cells (5×10^5 /well in a 6-well plate) were incubated for 24 h in serum-free RPMI-1640 medium containing 1% penicillin and streptomycin at 37 °C in 5% CO₂ for synchronization of the cell cycle. Cells were then treated for 24 h with PBIT or Se-PBIT at concentrations of 1.25, 2.5, or 5.0 μ M. Cells were harvested by scraping, centrifuged at 5 °C for 3 min at 1200 rpm, and washed once with cold PBS. The cells were then fixed with 70% cold ethanol (kept at –20 °C), while vortexing. The cells were placed at –20 °C until DNA staining. Prior to staining, the cells were centrifuged at 5 °C for 3 min at 1200 rpm. The cells were stained with 1 ml of hypotonic DNA staining buffer (100 μ g/ml propidium iodide, 200 μ g/ml ribonuclease A, 0.1% sodium citrate, and 0.3% Triton X-100) and vortexed. The cells

were covered and incubated at 4 °C for 1 h. Stained cells were analyzed by flow cytometry (Becton Dickinson FACScan). For each sample, 15,000 events were assessed, collected and analyzed for cell-cycle distribution. Percentage of cells in G0/G1, S, and G2/M were determined using ModFit LT™ software.

2.7. Western blot analysis

NCI-H460 and A549 cells treated with PBIT and Se-PBIT (5.0, 10 and 20 µM) for 24 h, were harvested by scrapping and washed with phosphate buffered saline. Cellular proteins were isolated with cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and freshly added 1 mM PMSF (Cell Signaling, Beverly, MA). Equal amounts of protein (30 µg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Antibodies used for Western blots were iNOS and COX-2 from Cayman Chemicals, Ann Arbor, MI, p53, p27, p21, PLA2 and actin from Santa Cruz, Santa Cruz, CA, and p38 and cleaved PARP from Cell Signaling Technology, Beverly, MA. Band expressions were developed using ECL reagents from Amersham, Piscataway, NJ.

2.8. Statistical evaluation

All assays (iNOS protein expression, NO production, cell growth inhibition, apoptotic induction) were performed at least three times and levels of all parameters measured were expressed as mean ± standard error (S.E.). Statistical comparisons between cells treated with PBIT and Se-PBIT versus control were based on *t*-test.

3. Results

3.1. The effect of PBIT and Se-PBIT on iNOS protein expression and nitric oxide production in A549 cells

Fig. 2A is a representative Western blot of iNOS protein expression at 5, 10 and 20 µM concentration of PBIT and Se-PBIT. Fig. 2B is the graphical presentation of mean densitometric measurements of bands from three Western blot assays. Both PBIT and Se-PBIT significantly reduced the level of iNOS protein at concentrations of 10 and 20 µM, whereas only Se-PBIT significantly reduced the level of iNOS at the lowest concentration of 5 µM. Fig. 2C is the graphical presentation of total nitrite production at 1, 5 and 10 µM concentrations of PBIT and Se-PBIT. Under experimental conditions employed in this study, PBIT at 1, 5, and 10 µM reduced the levels of NO, but not significantly. Se-PBIT, only at 10 µM, significantly ($P < 0.05$) reduced the levels of NO in A549. Collectively, these results indicate that, Se-PBIT and PBIT are weakly but equally effective inhibitors of iNOS protein expression and NO production.

3.2. The effect of PBIT and Se-PBIT on cell growth using cancer cells (A549, NCI-H460) and normal lung fibroblast cells

At doses up to 5.0 µM, PBIT had no effect on the cell growth in both cancer and normal cell lines whereas Se-PBIT reduced cell growth significantly at 2.5 and 5 µM concentration in both cancer cell lines but had no effect on normal lung fibroblast cells at all doses employed in this study (Fig. 3). The IC₅₀ of Se-PBIT is 3.0 and 2.5 µM in H460 and A549, respectively. Clearly, substitution of sulfur in PBIT by selenium, enhanced cell growth inhibition in lung cancer cell lines.

3.3. The effect of PBIT and Se-PBIT on the induction of apoptosis using A549 and NCI-H460 cells

Fig. 4 depicts the inductive effects of PBIT and Se-PBIT on apoptosis by ELISA in both cell lines. At doses up to 5 μ M PBIT failed to induce apoptosis in both cell lines. Se-PBIT significantly induced apoptosis at 1.0, 2.5 and 5.0 μ M concentrations in both cell lines. In H460 the elevation of apoptotic index is as high as seven-fold by Se-PBIT and in A549 it is more than nine-fold. It is clear from the results that Se-PBIT is a superior inducer of apoptosis to PBIT. PARP cleavage protein expression by PBIT and Se-PBIT in both cell lines was also examined. As evident from the blot, Se-PBIT induced intense bands at 5.0, 10.0 and 20.0 μ M in both cell lines (Fig. 4).

3.4. The effect of PBIT and Se-PBIT on the cell cycle using A549 and NCI-H460 cells

The effect of PBIT and Se-PBIT on FACS cell cycle analysis of H460 and A549 is shown in Fig. 5. In H460 cell line, doses up to 5.0 μ M of PBIT have no effect on the cell cycle whereas Se-PBIT changes the cell cycle pattern and the effect was dose-dependent; at G1 stage Se-PBIT increases the percentage from 42% to 58% and at G2–M stage it increases from 14% to 41%. In A549 cells both PBIT and Se-PBIT change the cell cycle pattern although Se-PBIT causes much more dramatic changes at both G1 and G2–M stage.

3.5. The effect of PBIT and Se-PBIT, using Western blot analysis, on protein expression in A549 and NCI-H460 cells

Western blot analysis of protein expression in A549 cell lines treated with varying concentrations of PBIT, and Se-PBIT is shown in Fig. 6. Although PBIT has no significant effect on these molecular markers, it is clearly evident that only Se-PBIT is capable of inducing p38, p27 and p21 which are important proteins known to play a major role in cell cycle regulation and apoptosis. We also showed that Se-PBIT is capable of inducing p53 protein expression in A549 cells. Although Se-PBIT inhibits PLA2 protein expression at higher doses it had no effect on COX-2 protein expression. Similar results were observed in the H460 cell line (data not shown).

4. Discussion

The results of this study demonstrate that, Se-PBIT and PBIT are weakly but equally effective inhibitor of NO production. In addition, both compounds had no effect on cell growth using normal lung fibroblast cells. However, we showed that selenium substitution of sulfur in PBIT clearly enhanced growth inhibition in two cancer cell lines, and is a superior inducer of apoptosis to that of PBIT. In contrast to PBIT, we showed that Se-PBIT in a dose-dependent manner induces distinct changes in cell cycle pattern. Critical molecular targets of lung cancer based on protein levels, were altered in a manner that may account for the effect of Se-PBIT on cell growth inhibition and the induction of apoptosis in these cell lines.

Since current strategies, including conventional therapies and clinical chemoprevention trails [3–6] to prevent lung cancer remain disappointing, it is of paramount importance to develop chemopreventive agents that target cellular processes and molecular events that are critical in the development of lung cancer. Epidemiological observation, preclinical studies and some clinical intervention trials provide support for the protective role of selenium against cancer, including lung cancer [17,18]. It has been conclusively proven that the structure (form) and dose are the most important factors that determine the chemopreventive efficacy of the selenium containing compounds [19]. Thus, in this study our goal was to develop novel synthetic organoselenium compounds and evaluate their efficacy in preclinical studies initially *in vitro* cell culture models.

As iNOS protein has been detected in both premalignant and malignant clinical biopsies from human lung tumors as well as elevated NO level [20–24], an iNOS inhibitor may be a potential candidate against lung tumorigenesis. It has been reported that genetic ablation of inducible NO synthase decreases mouse lung tumorigenesis [25]. Mice null for iNOS expression develop 83% fewer lung tumors than their wild-type littermates [25]. We chose an established chemopreventive agent that is known to be a relatively specific iNOS inhibitor, PBIT (Fig. 1), which contains sulfur atoms. Literature data also suggests that the inhibition of iNOS is due to several factors including the binding of the S-atom in PBIT and related analogs to the active site of the enzyme [11]. Since previous studies suggest that selenium substitution in established chemopreventive sulfur-containing compounds may result in more effective analogs, we synthesized and compared the effects of PBIT and Se-PBIT on cellular and molecular targets including iNOS expression that are known to be involved in the development of NSCLC.

In our previous report, we have shown that 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC), the most potent chemopreventive form of selenium examined in our laboratory, inhibits cell growth and induces apoptosis of NSCLC cells H460 and A549 using assays identical to those used in the present study [16]. Although *p*-XSC significantly induced apoptosis in both cell lines at 2.5 and 5.0 μ M, the apoptotic index was only two-fold higher than the control. By comparison, under identical conditions using the same lung cancer cell lines, Se-PBIT is proved to be a more effective organoselenium compound than any other selenium compounds tested so far in our laboratory as an inhibitor of cell growth and inducer of apoptosis. Moreover the effect of Se-PBIT on cell growth is specific to cancer cells only, while it had no effect on normal lung fibroblast cells at equal doses.

Based on the available knowledge in the literature we focused on target genes that are critical in the development of lung cancer. Specifically, we focused on gene products related to cell proliferation, apoptosis and cell cycle namely p53, p38, p27, p21, PLA2, COX-2 and iNOS. To elucidate the molecular targets of Se-PBIT in NSCLC, which may be responsible for its apoptotic induction and cancer cell growth inhibition, we have examined several protein expressions as described above and compared PBIT and Se-PBIT. Only Se-PBIT is capable of inducing p53, p38, p27 and p21 whereas PBIT had no effect in both cell lines even at a dose as high as 20.0 μ M.

p21 and p27 act as inhibitors to cyclin-dependent kinases (CDK) which in turn prohibit the activation of cyclin/CDKs complex formation leading to cell cycle progression. The CDK inhibitor p21 (also known as WAF1, CIP1) inhibits progression through cell cycle via several mechanisms. It can inhibit the cyclin D/CDK4 and cyclin E/CDK2 complexes early in G1 and it can also inhibit the cyclin A/CDK2 complex later, prior to the S phase/G2 phase transition [26]. Literature data indicates that inorganic selenite causing S-phase arrest is associated with decreased expression of p27kip1 and p21cip1 [27,28], while certain organoselenium compounds arrested cell cycle at G1 stage [27,29,30]. In the present study, simultaneous G1 and G2–M arrest by Se-PBIT in both H460 and A549 was observed.

p21 has been demonstrated to be a prognostic indicator of NSCLC survival. It has been shown that among NSCLC patients, cases with p21 overexpression have better mean survival time and 5 year survival rate than p21 negative cases [31,32]. It has been shown that p27 expression is reduced in NSCLC cases compared to normal counterparts [33]. Literature data also demonstrated that p27 expression is lost in some NSCLC cases especially in smokers [34]. In addition it has been reported that p38 activating genes have reduced expression in NSCLC [35].

Both A549 and H460 have wild type p53, and is weakly expressed in both cell lines as shown in Fig. 6. In this study we showed that only Se-PBIT is capable of inducing p53 protein expression. In addition to its effects on cell-cycle arrest, p53 can induce the apoptosis pathway through induction of Bax. It has been proposed recently that active p38 Map kinase promotes a G2–M cell cycle checkpoint through the phosphorylation and activation of p53 [36]. It is well known that post-translational stabilization of p53 is achieved through phosphorylation at multiple sites by protein kinases including p38 [37,38]. It has been reported that the induction of apoptosis and inhibition of cell growth have been linked to the activation of p38 [39,40]. Recently it was reported that inorganic sodium selenite activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells [41].

Although only Se-PBIT is a potent inducer of apoptosis in NSCLC cell lines, we could not establish any significant difference in its effect on NO production or iNOS expression with its parent compound PBIT. Thus, apoptotic effect of Se-PBIT in both cancer cell lines could not only be dependent on iNOS inhibition and future studies are required to provide insights into its mechanism of action.

Overall we have developed a novel selenium-containing compound (Se-PBIT) that is superior to an established sulfur-containing chemopreventive agent (PBIT) as an inhibitor of cell growth and an inducer of apoptosis; only Se-PBIT altered certain proteins that are known to be important targets in the development of NSCLC. Although, the mechanisms that can fully account for these effects remain to be determined, the results are encouraging to further evaluate the chemopreventive efficacy of Se-PBIT against the development of NSCLC in a well-defined animal model.

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The synthesis of PBIT and Se-PBIT and FACS analysis were performed in the Organic Synthesis and Flow Cytometry Facilities, respectively of the Penn State Cancer Institute, Penn State College of Medicine.

Abbreviations

CDK	cyclin dependent kinases
COX-2	cyclooxygenase-2
iNOS	inducible nitric oxide synthase
MTT	methylthiazoletetrazolium
NO	nitric oxide
PBIT	S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiurea
PLA2	phospholipase A2
p-XSC	phenylenebis(methylene)selenocyanate
Se-PBIT	Se,Se'-1,4-phenylenebis(1,2-ethandiyl)diisoselenoureadihydrobromide

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Fig. 1.
Schematic presentation of the synthesis of PBIT and Se-PBIT.

(A) A549 iNOS Protein Expression

(C)

Fig. 2.

The effect of PBIT and Se-PBIT on iNOS protein expression and nitric oxide production in A549 cells. (A) A representative Western blot protein expression of iNOS at 5.0, 10.0 and 20 μ M doses of PBIT and Se-PBIT. Cells were pretreated with cytomix (10 ng/ml IL-1 β , 10 ng/ml TNF- α and 100 ng/ml IFN- γ) and starved of FBS for 24 h before treatment. The control band in A is iNOS protein expression from total cell protein lysate from A549 cells that had not been treated with any compound. Actin was used as a housekeeping gene for protein loading control and was further used to normalize iNOS protein expression across samples. Relative band densities after actin normalization were used to construct the bar graph. (B) Bar diagram of the effect of PBIT and Se-PBIT on iNOS protein expression from densitometric measurement of three Western blots from three separate treatments. (*) Statistically significant from the control treatment, $P < 0.05$. (C) Bar diagram of the effect of PBIT and Se-PBIT on the total nitrite production from calorimetric measurement of three separate treatments. (*) Statistically significant ($P < 0.05$, t -test) from control treatments.

Fig. 3.

The effect of PBIT and Se-PBIT on cell growth using NCI-H460, A549 and normal lung fibroblast cells as determined by MTT assay. Percentage of control was quantitated from three independent experiments performed in triplicate. Mean of three values was determined and the results are expressed as percent of control. Data are presented as mean $\pm$ S.E. (*) Statistically significant ($P < 0.05$, t -test) as compared with untreated controls.

Fig. 4.

The effect of PBIT and Se-PBIT on the induction of apoptosis using NCI-H460 and A549 as determined by cell-death-ELISA and protein expression of cleaved PARP analysis. Actin was used as a housekeeping gene for protein loading control. Percentages of apoptotic cells were quantitated from three independent experiments. The assay was performed in triplicate and the results are presented as enrichment factor (ratio of treatment over control). Data are presented as mean $\pm$ S.E. (*) Statistically significant ($P < 0.05$, t -test) as compared with untreated controls.

Fig. 5.

Bar diagram of the effect of PBIT and Se-PBIT on cell cycle analysis using H460 and A549 cells from flow cytometric measurements at doses 1.25, 2.5 and 5.0 μM . In each triplet of bars the 1st bar represents the percentage of cells in stage G1, the 2nd bar represents the percentage cells in stage S and the 3rd bar represents the percentage cells in G2–M stage.

Fig. 6. The effect of PBIT and Se-PBIT, using Western blot analysis on cPLA2, COX-2, p53, p38, p27 and p21 in A549 cells. Similar results were obtained in H460 cells.