PBISe, a novel selenium-containing drug for the treatment of malignant melanoma

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Abstract

Malignant melanoma is the most deadly form of skin cancer due to its highly metastatic nature. Untargeted therapies are ineffective for treating metastatic disease, leading to the development of agents specifically inhibiting proteins or pathways deregulated in melanoma. The deregulation of inducible nitric oxide synthase (iNOS) is one such event occurring in melanoma, and is correlated with poor survival. Current iNOS inhibitors, such as PBIT [*S,S*′-1,4-phenylenebis(1,2-ethanediyl)bis-isothiourea], require high concentrations for clinical efficacy causing systemic toxicity. To develop more potent agents effective at significantly lower concentrations, a novel isosteric analogue of PBIT was synthesized, called PBISe [*S,S*′-1,4-phenylenebis(1,2-ethanediyl)bis-isoselenourea], in which sulfur was replaced with selenium. PBISe kills melanoma cells >10-fold more effectively than PBIT, and cultured cancer cells are 2- to 5-fold more sensitive than normal cells. Like PBIT, PBISe targets iNOS but also has new inhibitory properties acting as an Akt3 pathway inhibitor and mitogen-activated protein kinase (MAPK) cascade activator, which causes decreased cancer cell proliferation and increased apoptosis. Inhibition of cellular proliferation mediated by PBISe induced a G_2-M phase cell cycle block linked to excessively high MAPK activity causing decreased cyclin D1 and increased p21 as well as p27 levels. PBISe promotes apoptosis by inhibiting Akt3 signaling, elevating cleaved caspase-3 and PARP levels. Compared with PBIT, PBISe reduced tumor development by 30% to 50% in mice inducing a 2-fold increase in apoptosis with negligible associated systemic toxicity. Collectively, these results suggest that PBISe is a potent chemotherapeutic agent with novel properties enabling the targeting of iNOS, Akt3, and MAPK signaling, thereby promoting melanoma cell apoptosis and inhibition of proliferation. [Mol Cancer Ther 2008;7(5):1297–308]

Introduction

Of the three main forms of skin cancer, malignant melanoma has the most significant effect on human health and carries the highest risk of mortality from metastasis (1). Nontargeted therapeutics ranging from surgery to immunotherapy, radiotherapy, and chemotherapy have all failed to provide effective long-term treatment for patients suffering from advanced stages of this disease (2, 3). Thus, the 2007 prognosis for patients with metastatic disease remains very poor, with average survival ranging from 6 to 10 months (4). In spite of the widely appreciated magnitude of the problem, there is still a critical gap in the development of chemotherapeutics targeting proteins or signaling pathways whose deregulation contributes to melanoma development (4, 5).

Inducible nitric oxide synthase (iNOS) is a calcium-independent, cytokine-inducible enzyme involved in the production of a bioactive, pleiotropic regulatory and signaling molecule from arginine called nitric oxide (6). Recent studies have implicated iNOS as a potential therapeutic target in melanoma (7–10). Among the three isoforms of NOS, calcium-independent iNOS produces high levels of nitric oxide, promoting the development of a malignant phenotype (6–8, 10). Elevated iNOS expression/activity is also correlated with poor survival rates of patients with melanoma (7, 8). A number of small molecule iNOS inhibitors have been identified and tested *in vitro* and *in vivo* (6, 11–13). *In vitro* studies using iNOS inhibitors, *S*-methylisothiourea and aminoguanidine, have shown the inhibition of nitric oxide production and induction of apoptosis mediated by caspase-1/3 and PARP cleavage (10). PBIT [*S,S*′-1,4-phenylenebis(1,2-ethanediyl)bis-isothiourea] is an iNOS-selective inhibitor that is effective in preventing colon and esophageal cancer in rats following dietary administration (13–15). However, due to its low potency, poor cell permeability, and associated systemic toxicity, its use in clinical settings is questioned (13–15).

To create a more potent compound that would be an effective melanoma inhibitor at significantly lower concentrations, a novel isosteric analogue of PBIT was synthesized, called PBISe [*S,S*′-1,4-phenylenebis(1,2-ethanediyl)bis-isoselenourea], in which sulfur was replaced with selenium. Selenium is a trace element required for the protection of

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cell membranes, and is a component of various biomolecules (16, 17). Low selenium levels occur in serum isolated from patients suffering from a variety of different cancer types including melanoma (18). Furthermore, dietary selenium supplementation can reduce tumor growth as well as improve quality of life for patients (18–22).

Incorporation of selenium into the structure of PBIT has potential to provide the agent with novel tumor-inhibitory properties, due to the presence of selenium as well as structure-activity relationships associated with the compound (13, 23, 24). Specifically, selenium has inhibitory effects on phosphorylation of the survival kinase Akt in the phosphoinositide-3-kinase (PI3K) signaling pathway, thereby inhibiting its activity and that of the downstream signaling cascade (25). Akt kinase family members Akt1/PKBα, Akt2/PKBβ, or Akt3/PKBγ play important roles in the development of a large variety of cancers including melanoma (26, 27). Therefore, a novel compound inhibiting iNOS and Akt signaling might be a more effective tumor inhibitor (8, 10).

Among the three Akt family members, Akt3 is the predominantly active isoform in ~70% of melanomas (27). Akt3 activity promotes tumorigenesis by signaling through PRAS40 to decrease the apoptotic sensitivity of melanoma cells (26–29). Reduction of Akt3 or PRAS40 activity using small interfering RNA or through PTEN expression significantly decreased the tumorigenic potential of melanoma cells by increasing cellular apoptotic sensitivity (28, 29). Therefore, the Akt3 signaling cascade is one of the major pathways important in melanoma and inhibiting it could be therapeutically important for treating this disease.

Constitutive activation of the mitogen-activated protein kinase (MAPK) pathway (Ras/Raf/Mek/Erk) through Ras mutations in 10% to 15% and B-Raf mutation in ~60% of melanomas is a second important signaling cascade deregulated in melanomas (30). Activating B-Raf mutations are acquired, somatic, postzygotic events and are not inherited in families (31). Among different B-Raf mutations, a single-base missense substitution (T to A at nucleotide 1799) that changes valine to glutamic acid at codon 600 (V600E) in exon 15 is prevalent in ~90% of melanoma tumors with a mutation of B-Raf (32). ^{V600E}B-Raf protein leads to kinase activity 10.7 times higher than occurs in normal cells and causes hyperactivity of the MAPK pathway (32).

Treatment of cells with PBIT to inhibit iNOS increases MAPK pathway activity leading to higher levels of active phosphorylated Erk1/2 and increased expression of downstream iNOS (33, 34). Abnormally high activation of the MAPK pathway can inhibit cellular growth in a wide variety of normal and cancer cells by promoting cellular senescence, including melanomas (35). Recent evidence suggests that constitutively active ^{V600E}B-Raf initially promotes nevi development during melanoma tumor progression resulting in high, intense activation of the MAPK pathway, which is inhibitory, and that Akt3 activity is required to phosphorylate^{V600E}B-Raf in order to reduce its activity and the MAPK pathway activity to levels promoting rather than inhibiting proliferation (36). Thus, increasing MAPK pathway activity following PBIT treatment or by inhibiting Akt3 could inhibit tumor cell proliferation and thereby play therapeutically important roles for the treatment of melanoma.

In this study, we show that PBISe but not PBIT effectively kills melanoma cells and that cultured cancer cells are 2- to 5-fold more sensitive to the compound than normal cells. Although PBISe targets iNOS in a manner similar to PBIT, it also has new inhibitory properties acting as an Akt3 signaling cascade inhibitor and MAPK pathway activator. These novel properties lead to decreased cell proliferation and increased apoptosis of melanoma cells. Only PBISe but not PBIT inhibits cancer cell proliferation by blocking cultured cells in the G_2-M phase of the cell cycle and decreases cyclin D1 expression with corresponding increases in p21 and p27 levels. Furthermore, PBISe but not PBIT promotes apoptosis in melanoma cells through the inhibition of Akt3 signaling leading to increased levels of cleaved caspase-3/7 and PARP. Compared with PBIT, PBISe reduced tumor development by 30% to 50% in mice with negligible associated toxicity. Mechanistically, tumors undergo 2- to 3-fold more apoptosis following treatment with PBISe compared with PBIT. Thus, PBISe is a more potent chemotherapeutic agent than PBIT.

Materials and Methods

Cell Lines and Culture Conditions

The human metastatic melanoma cell lines UACC 903 and 1205 Lu, normal human fibroblast cells (FF2441), were maintained in DMEM (Invitrogen) and supplemented with 10% fetal bovine serum (FBS; Hyclone). The vertical growth phase melanoma cell line WM115 was maintained in Tu2% medium lacking calcium chloride, but supplemented with 2% heat-treated FBS (56°C for 30 min) and L-glutamine (Mediatech; ref. 27). Colon adenocarcinoma cell line Caco-2 was grown in Advanced DMEM supplemented with 10% heat-inactivated FBS (56°C for 30 min) and 2 mmol/L of L-glutamine.

PBIT and PBISe Synthesis

PBIT and PBISe was synthesized as reported (13).[7] The methyl ester of 1,4-phenylenediacetic acid was reduced with lithium aluminum hydride to obtain a diol, which upon bromination with carbon tetrabromide and triphenylphospine, resulted in the generation of a dibromo derivative. This was reacted with thiourea to generate PBIT. Similarly, PBISe was synthesized by reacting a dibromo compound with selenourea instead of thiourea.[7] Monosubstituted derivatives *S*-phenylethyl isothiourea (PEIT) and *S*-phenylethyl isoselenourea (PEISe) were synthesized by reacting phenyl ethylbromide with thiourea

[7] Desai D, Madhunapantula SV, Gowdahalli K, El-Bayoumy K, Robertson GP, Amin S. Novel organoselenium analog of iNOS inhibitor for cancer therapy. Submitted for publication.

or selenourea, respectively.[7] The purity of PBIT and PBISe were confirmed by high-performance liquid chromatography and structure characterized using proton nuclear magnetic resonance (500 MHz, Bruker; Bruker Optics, Inc.) and mass spectrometry (Finnigan Mat95).[7] High-performance liquid chromatography analysis showed that PBIT and derivatives PBISe, PEIT, and PEISe were 99.5% pure. Results were PEIT δ: 7.32 to 7.24 (m, 5H, aromatic), 3.34 (t, 2H, J = 7.0 Hz, CH_2-Ph), 2.99 (t, 2H, J = 7.0 Hz, CH2-S), m/z ion intensity −181 (M^+, 100), 159 (10), 105 (10); PEISe: 7.29 to 7.20 (m, 5H, aromatic), 3.02 (t, 2H, J = 7.0 Hz, CH_2-Ph), 3.48 (t, 2H, J = 7.0 Hz, CH_2-Se), m/z ion intensity −229 (M+, 100), 181 (80), 159 (10), 105 (10); PBIT: δ: 7.25 (s, 4H, aromatic), 3.37 (t, 4H, J = 7.2 Hz, Ph-CH2), and 3.0 (t, 4H, J = 6.86 Hz, C-CH2), m/z ion intensity 283 (M^{+1}, 100), 266 (40), 207 (30), 142 (70), 131 (100), 104 (10), 77 (30); and PBISe: δ: 7.25 (s, 4H, aromatic), 3.37 (t, 4H, J = 7.2 Hz, Ph-CH2), and 3.21 (t, 4H, J = 6.86 Hz, C-CH2), m/z ion intensity 377 and 379 (M^{+1}, 50), 335 and 337 (80), 253 and 255 (70), 188 and 190 (100), 131 (50), 73 (10). Furthermore, electrospray-mass spectrometry showed an observed molecular weight close to the calculated mass. For example, the calculated m/z for PBISe was 378.9934 and the observed m/z was 378.9935.

Western Blot Analysis

Cell lysates were harvested by the addition of lysis buffers containing 50 mmol/L of HEPES (pH 7.5), 150 mmol/L of NaCl, 10 mmol/L of EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L of sodium orthovanadate, 0.1 mmol/L of sodium molybdate, 1 mmol/L of phenylmethylsulfonyl fluoride, 20 μg/mL of aprotinin, and 5 μg/mL of leupeptin. Whole cell lysates were centrifuged (≥10,000 × g) for 10 min at 4°C to remove cell debris. Protein concentrations were quantitated using the bicinchoninic acid assay (Pierce), and 30 μg of lysate loaded per lane onto NuPAGE Gels (Life Technologies). Following electrophoresis, samples were transferred to polyvinylidene difluoride membrane (Pall Corporation). Blots were probed with antibodies to PRAS40 and phosphorylated PRAS40 (Thr^{246}; Invitrogen); antibodies to iNOS, cyclin D1, p27, p21, Erk2, α-enolase, and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology; and antibodies to Akt3, phosphorylated-Akt (Ser^{473}), phosphorylated-Erk 1/2 (Thr^{202}/Tyr^{204}), caspase-3 and cleaved PARP were from Cell Signaling Technology. Immunoblots were developed using enhanced chemiluminescence (Pierce).

Cell Viability, Proliferation, Apoptosis, and Cell Cycle Analysis

The viability and IC_{50} of melanoma cells following treatment with PBIT, PBISe, PEIT, and PEISe were measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). A total of 5 × 10^3 cells/well in 100 μL of DMEM-10% FBS were grown in a 96-well plate for 36 or 72 h, respectively, for melanoma (UACC 903, 1205 Lu, and WM115) and human fibroblast (FF2441) cell lines, and treated with either PBS vehicle or increasing concentrations (3.5–21 μmol/L) of PBIT, PBISe, PEIT, or PEISe for 24 h. Cellular viability compared with control-treated cells was measured using the MTS assay. IC_{50} (μmol/L) values for each compound in respective cell lines was determined from three independent experiments using GraphPad Prism version 4.01 (GraphPad).

Cellular proliferation and apoptosis rates were measured by seeding 5 × 10^3 cells in 96-well plates, followed by treatment for 24 h with each respective agent. Proliferation and apoptosis rates were measured using a bromodeoxyuridine ELISA kit (Roche Applied Sciences) or Apo-ONE homogenous caspase-3/7 assay kit (Promega), respectively (29).

Cell cycle analysis was undertaken by growing 1.5 × 10^6 melanoma cells in 100-mm culture dishes followed by treatment with PBIT or PBISe for 24 h. Cells were collected and stained using propidium iodide (100 μg/mL; Sigma), 20 μg/mL of RNase A (Roche), and 3 μg/mL of Triton X-100 dissolved in 0.1% (W/V) sodium citrate for 30 min at 4°C (37). Stained cells were analyzed using the FACScan analyzer (Becton Dickinson) and data processed using ModFit LT software (Verity Software House).

Nitrite Estimation

Nitrite content in the cell culture medium was determined using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company). Caco-2 cells (1 × 10^6) were plated in 60-mm culture dishes in advanced DMEM containing 10% heat-treated FBS for 12 h, and growing cells conditioned with phenol red–free DMEM containing 0.5% FBS for an additional 12 h. Cells were then treated with increasing concentrations of PBIT (40 and 80 μmol/L) or PBISe (2 and 4 μmol/L) dissolved in phenol red–free DMEM (2 mL) containing 0.5% FBS for 72 h. Culture supernatants were collected and total nitrite (nitrate + nitrite) measured by incubating 80 μL of the supernatant with an enzyme cofactor mixture (10 μL) and a nitrate reductase (10 μL) for 2 h. Total nitrite was measured by the addition of Griess reagent I and II (50 μL each). A nitrate standard curve (5–35 μmol/L) was simultaneously prepared. The total nitrite present in the medium alone was subtracted from all experimental values and represented as micromolar nitrite produced following each treatment.

Tumorigenicity Assessments and Measurement of Proliferation and Apoptosis Rates

Tumor kinetics were measured by s.c. injection of 5 × 10^6 UACC 903 or 2.5 × 10^6 1205 Lu cells in 0.2 mL of DMEM containing 10% FBS above the left and right rib cages of 4- to 6-week-old female nude mice (Harlan Sprague-Dawley). Six days later, mice were randomly placed in control DMSO and experimental (PBIT and PBISe) groups (n = 5 animals; 10 tumors total) followed by i.p. treatment with PBIT (0.315 μmol/L) or PBISe (0.315 μmol/L; equivalent to 2.5 ppm selenium/20 g mouse) on Monday, Wednesday, and Friday for 3 weeks. The dimensions of the developing tumors and body weights were measured.

For mechanistic studies, 5 × 10^6 UACC 903 cells were injected s.c. into nude mice, generating tumors of the same size developing at parallel time points. Six days later, mice

were treated i.p. with DMSO vehicle, PBIT, or PBISe (0.315 μmol/L each) on alternate days up to day 15. Size- and time-matched tumors were harvested at days 9, 11, 13, and 15 to assess changes in cell proliferation and apoptosis. A small portion of tumor was flash-frozen in liquid nitrogen for caspase-3/7 activity analysis and processed by pulverizing into powder and adding protein lysis buffer [600–800 μL/50 mg powder, 50 mmol/L Tris-HCl (pH 7.5) containing 0.1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L sodium fluoride, 10 mmol/L sodium β-glycerol phosphate, 5 mmol/L sodium PPi, 1 mmol/L activated sodium orthovanadate, protease inhibitor cocktail, and 0.1% (v/v) 2-mercaptoethanol] followed by repeated centrifugation (10,000 × *g*). Protein concentration was quantitated using Bio-Rad protein assay reagent (Bio-Rad Laboratories) and analyzed by Western blotting. For caspase-3/7 activity determination, 100 μg of protein lysate was incubated with a rhodamine-110–conjugated caspase-3/7 substrate (Z-DEVD) for 1 to 2 h and fluorescence (485 nm excitation and 520 nm

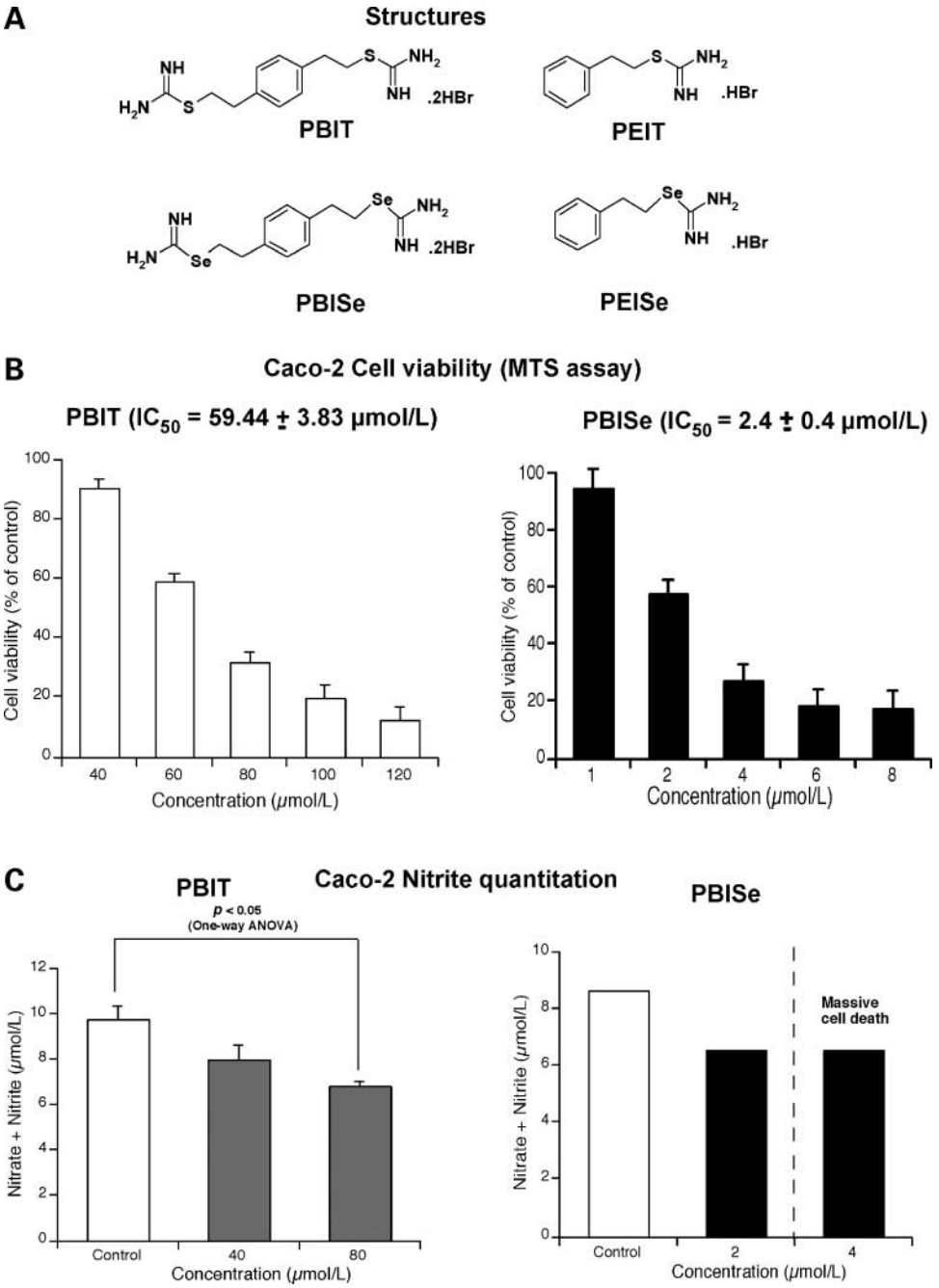
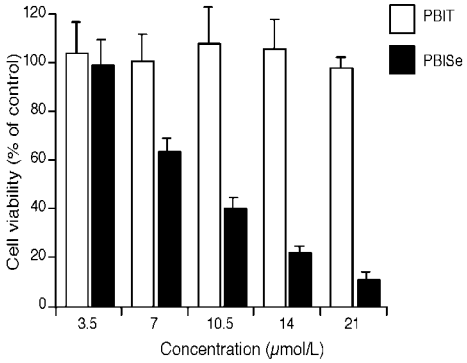


Figure 1. Characterization of PBISe as an iNOS inhibitor. **A,** chemical structures of PBIT, PBISe, PEIT, and PEISe. PBIT, a known iNOS-specific inhibitor, was chemically modified by replacing sulfur (S) with selenium (Se) to yield PBISe. PEIT and PEISe are monosubstituted forms of PBIT and PBISe, respectively. **B,** PBISe inhibits colon adenocarcinoma cell growth 25-fold more effectively than PBIT. Caco-2 colon adenocarcinoma cells (5×10^3) were grown in 96-well plates for 24 h and treated with increasing concentrations of PBIT (20–120 μmol/L) and PBISe (1–10 μmol/L) for 72 h. The number of viable cells was quantified using the MTS assay. PBISe (IC_{50} = 2.4 ± 0.4 μmol/L) more efficiently kills Caco-2 cells compared with PBIT (IC_{50} = 59.44 ± 3.83 μmol/L), indicating that substituting selenium for sulfur increases the compound's potency. **C,** PBIT and PBISe decrease total nitrite production in colon adenocarcinoma cells by inhibiting iNOS activity. iNOS activity in terms of total nitrate + nitrite produced was measured using a nitrate/nitrite colorimetric assay kit developed by Cayman Chemical Company. Caco-2 cells (1×10^6) were plated in 60 mm culture dishes for 24 h. Increasing concentrations of PBIT (40 and 80 μmol/L) or PBISe (2 and 4 μmol/L) in phenol red–free DMEM (2 mL) containing 0.5% FBS, were added. Amount of total nitrate + nitrite in culture supernatant was measured 72 h later. Treatment with 80 μmol/L of PBIT or 2 μmol/L of PBISe significantly reduced iNOS activity compared with control PBS-treated cells ($P < 0.05$; one-way ANOVA). However, a further increase in PBISe concentration (4 μmol/L) resulted in massive cell death, preventing the estimation of nitrite levels.

A **UACC 903 cell viability (MTS assay)**

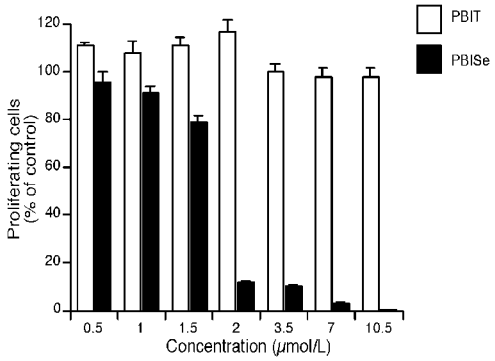


B **Comparison of PBISe IC_{50} (μmol/L) values between melanoma cells and normal human fibroblasts**

Treatment time (hours)	Human fibroblast cells (FF2441)	Human metastatic melanoma cells (UACC 903)	Fold Difference
4	77.76	37.64	2.06
6	75.85	14.71	5.16
12	26.59	11.58	2.29
24	17.08	7.78	2.19

PBISe inhibits melanoma proliferation at concentrations 2-5 fold lower than human fibroblast cells

C **UACC 903 BrdU incorporation**



D **UACC 903 Caspase-3/7 activity**

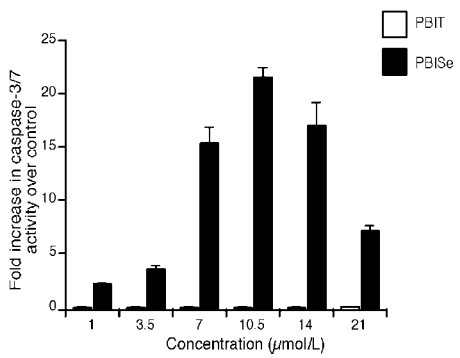


Figure 2. PBISe inhibits melanoma cell viability at 2- to 5-fold lower concentrations than normal human fibroblast cells. **A,** PBISe more effectively kills melanoma cells that PBIT. Human metastatic melanoma cell line UACC 903 (5×10^3) was treated with increasing concentrations (3.5 – 21.0 μmol/L) of PBIT or PBISe for 24 h. Cell viability was quantified by MTS assay and dose-response curves plotted. *Columns,* average of percent cell viability calculated from three independent experiments; *bars,* SE. PBISe (IC_{50}; 8.1 μmol/L) more effectively inhibit melanoma cell viability compared with PBIT or monosubstituted PEIT and PEISe (IC_{50} > 100 μmol/L). **B,** PBISe more efficiently inhibits melanoma cell growth compared with normal human fibroblast cells. Normal human fibroblasts (5×10^3; FF2441) and metastatic melanoma cells (UACC 903) were plated in 96-well plates in 100 μL of DMEM containing 10% FBS and grown for 72 and 36 h, respectively. Exponentially growing cells were treated with increasing concentrations (2.5 – 100 μmol/L) of PBISe for 4, 6, 12, and 24 h and IC_{50} (μmol/L) values determined. PBISe inhibits melanoma cell growth at concentrations 2- to 5-fold lower than fibroblast cells at all time points. **C,** PBISe but not PBIT inhibits melanoma cell proliferation. UACC 903 cells (5×10^3) were treated with increasing concentrations of PBISe and PBIT (0.5 – 10.5 μmol/L), and proliferating cells were measured using bromodeoxyuridine to label cells for 4 to 6 h. PBISe (2 μmol/L) reduced UACC 903 cellular proliferative capacity by ~90% compared with PBS vehicle or PBIT-treated cells. Results represent the average of three independent experiments; bars, SE. **D,** PBISe treatment promotes melanoma cell apoptosis. UACC 903 cells (5×10^3) were plated and treated for 24 h with increasing concentrations of PBISe and PBIT (0.5 – 10.5 μmol/L). Levels of caspase-3/7 activity (an indicator of apoptosis) in cells exposed to PBIT, PBISe or PBS vehicle were measured using the Apo-ONE homogeneous caspase-3/7 assay kit. Results show fold increase in caspase-3/7 activity relative to PBS vehicle treated cells. *Columns,* average of three independent experiments; *bars,* SE. Compared with PBIT, increasing concentrations of PBISe elevated caspase-3/7 activity in a dose-dependent manner up to 10.5 μmol/L, which decreased at higher doses due to massive cell death (>80%), decreasing caspase-3/7 activity.

emission) measured in a SPECTRAmax M2 plate reader. Cell proliferation was measured in formalin-fixed, paraffin-embedded tumor sections using purified mouse anti-human Ki-67 from PharMingen. A minimum of six different tumors with four to six fields per tumor were analyzed and results represented as the average ± SE.

Systemic Toxicity Assessments

Four- to 6-week-old female nude mice (Harlan Sprague-Dawley) were injected i.p. with either control DMSO vehicle, PBIT, or PBISe (0.315 μmol/L each) for 3 weeks by following the regime used for tumorigenicity. Blood was then collected from each animal in plasma separator tubes with lithium heparin (BD Microtainer, BD) following cardiac puncture and analyzed for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, glucose, and creatinine to ascertain liver, heart, kidney, and pancreas-related toxicity. For morphologic examination of blood cells, whole blood was collected in microtainer tubes containing K_2EDTA (BD Microtainer, BD) and RBC, WBC, lymphocytes, monocytes, eosinophils, platelets, total hemoglobin, and hematocrit percentage were analyzed. Blood was also microscopically examined for segregates, polychromatin bodies, and smudge cells. A portion of the vital organs—liver, heart, kidney, intestine, pancreas, and adrenal glands—from each animal was formalin-fixed and paraffin-embedded to examine for toxicity-associated changes in cell morphology and organization following H&E staining.

Statistical Analysis

Statistical analysis was undertaken using the one-way or two-way ANOVA followed by Tukey's or Bonferroni's post hoc tests. Results were considered significant at $P < 0.05$.

Results

PBISe More Effectively Inhibits Colon Adenocarcinoma Cell Growth than PBIT

PBIT is a non–amino acid analogue of L-arginine that inhibits iNOS activity at rates 190- and 5-fold higher than eNOS or nNOS family members, respectively (6, 13). It prevents colon and esophageal tumor development in animals but has low potency and poor cell permeability, requiring high concentrations for efficacy with significant acute toxicity, reducing its clinical utility (13–15). To overcome these limitations, a novel derivative, called PBISe, was synthesized in which sulfur was replaced with selenium in order to increase the potency of the compound (Fig. 1A). Control monosubstituted sulfur and selenium compounds, PEIT and PEISe, were also synthesized to determine whether the disubstitution in the aromatic ring contributed to chemotherapeutic efficacy (Fig. 1A).

Because PBIT is a known iNOS-specific inhibitor, with demonstrated efficacy at reducing iNOS activity and colon adenocarcinoma cell growth, similar approaches to those reported in the literature were used to validate growth and iNOS-inhibitory effects mediated by PBIT on cultured Caco-2 cells and compared it to PBISe to confirm that the new compound acted in a manner similar to the original compound (14). Treatment with increasing concentrations of PBIT (40–120 μmol/L) for 72 h showed the predicted dose-response curve with a resulting IC_{50} of ~60 μmol/L (Fig. 1B). Using the same approach, PBISe was significantly more effective, having an IC_{50} of 2.4 μmol/L indicating a 25-fold increase in potency compared with PBIT (Fig. 1B). Thus, PBISe is a far superior and more potent inhibitor of colon adenocarcinoma cells than PBIT.

PBISe Inhibits iNOS Activity

To show that PBIT and PBISe similarly inhibited iNOS activity in terms of nitrite production, total nitrate + nitrite levels were measured in Caco-2 cells using a reported colorimetric assay (Fig. 1C; ref. 10). Results showed that 80 μmol/L of PBIT significantly reduced nitrate + nitrite levels compared with control PBS-treated cells, which was indicative of iNOS inhibition ($P < 0.05$; one-way ANOVA; ref. 10). Similarly, PBISe also reduced total nitrate + nitrite production in Caco-2 cells but at much lower concentrations of 2 to 4 μmol/L compared with PBIT (Fig. 1C). Unfortunately, higher concentrations could not be used because PBISe treatment killed >80% of cells at 2 to 4 μmol/L; therefore, total nitrate + nitrite levels regulated by live cells could not be measured. Thus, like PBIT, PBISe inhibited iNOS activity but also had additional inhibitory capabilities.

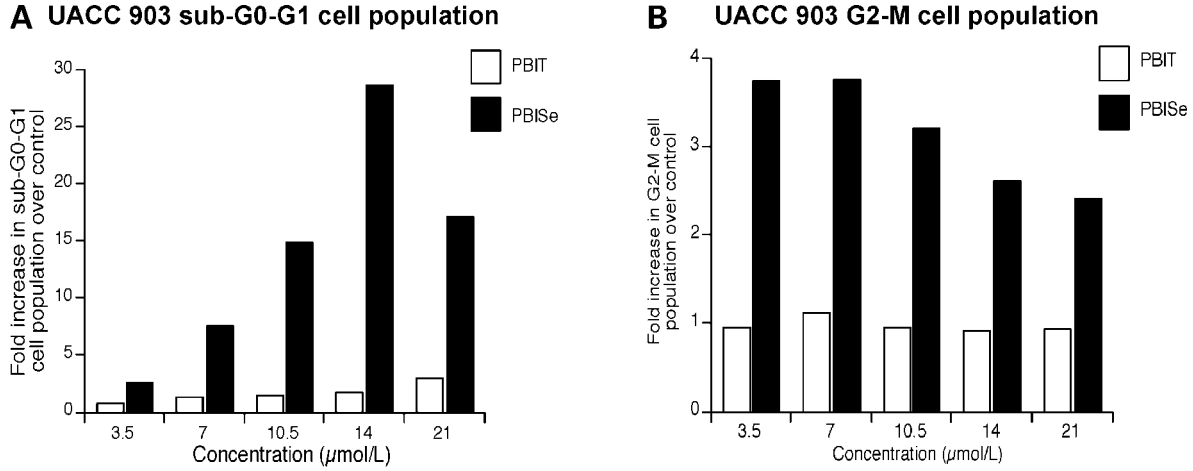
PBISe is >10-fold More Effective at Killing Melanoma Cells than PBIT and Inhibits Melanoma Cell Growth 2- to 5-fold More Effectively than Normal Cells

To compare the effectiveness of PBISe versus PBIT for killing melanoma cells, three independently derived melanoma cell lines, WM115, UACC 903, and 1205 Lu were treated with 3.5 to 21 μmol/L of PBISe or 10 to 100 μmol/L of PBIT, PEIT, and PEISe for 24 h and cell viability measured using the MTS assay. A representative example of the MTS analysis for UACC 903 is shown in Fig. 2A, demonstrating the effectiveness of PBISe for decreasing melanoma cell viability compared with PBIT. Dose-response curves for these agents were generated and IC_{50} values determined (Table 1). PBISe decreased the viability of all three melanoma cell lines with an IC_{50} range of 8 to 10 μmol/L (Table 1). In contrast, PBIT or control monosubstituted derivatives PEIT or PEISe had no effect at these concentrations with average IC_{50} values >100 μmol/L (Table 1). PEISe was moderately better with an IC_{50} of ~80 μmol/L for the WM115 cell line; however, because a similar trend was not observed for other cell lines, this

Table 1. PBISe IC_{50} (μmol/L) values for melanoma cell lines

Cell line	PBIT	PBISe	PEIT	PEIS
WM115	>100	9.7 ± 1.4	>100	~80
UACC 903	>100	8.1 ± 0.8	>100	>100
1205 Lu	>100	9.8 ± 0.7	>100	>100

NOTE: IC_{50} values show that PBISe but not PBIT or the corresponding monosubstituted PEISe or PEIT is effective at inhibiting melanoma cell viability at concentrations 10-fold lower than PBIT.



C Effect of PBISe and PBIT treatment on UACC 903 cell cycle

	DMSO	PBIT					PBISe				
Concentration (μmol/L)		3.5	7	10.5	14	21	3.5	7	10.5	14	21
G0-G1 (%)	58.0	59.40	60.3	60.1	62.4	63.0	52.7	47.0	48.0	53.0	53.0
S (%)	32.0	31.10	29.0	30.5	29.0	28.0	10.0	16.0	20.0	21.0	23.3
G2-M (%)	10.0	9.45	11.0	9.4	9.00	9.10	37.3	37.5	32.0	26.0	24.0

Figure 3. PBISe increased the sub – G_0-G_1 cell population and induces G_2-M cell cycle arrest in melanoma cells. **A,** PBISe treatment elevates the sub – G_0-G_1 cell population indicating increased cellular apoptosis. UACC 903 cells were treated with PBIT, PBISe, or PBS vehicle control, and 24 h later, total cells were stained with propidium iodide and analyzed for cell cycle distribution using a FACScan analyzer. PBISe treatment increased the sub – G_0-G_1 cell population (an indicator of apoptosis) compared with PBS- or PBIT-exposed cells. *Columns,* average of two independent experiments. **B,** G_2-M cell population increases 3- to 4-fold following PBISe exposure. UACC 903 cells treated with PBIT or PBISe (3.5 – 21 μmol/L), for 24 h were stained with propidium iodide and cell cycle stages analyzed using a FACScan analyzer. The G_2-M cell population increased from ~10% to ~37% following 3.5 to 21 μmol/L PBISe exposure. **C,** cell cycle analysis following PBISe exposure. UACC 903 cells treated with PBIT or PBISe (3.5 – 21 μmol/L) were stained with propidium iodide and cell cycle analysis carried out using a FACScan analyzer and a proportion of the cells in each phase of the cell cycle (G_0-G_1, S, G_2-M) estimated. A significant decrease in the G_0-G_1 and S phase cell population occurred following PBISe but not PBIT treatment of UACC 903 melanoma cells, which was accompanied by a G_2-M cell population increase from ~10% to ~37% following 3.5 to 21 μmol/L of PBISe exposure.

difference was considered negligible. Thus, PBISe was >10-fold more effective than PBIT, PEIT, or PEISe at killing all three melanoma cell lines.

Next, the sensitivity of melanoma cells to PBISe was compared with normal cells. Normal human fibroblast, FF2441, and UACC 903 cells were treated with 2.5 to 100 μmol/L of PBISe and IC_{50} measured at 4, 6, 12, and 24 h (Fig. 2B). Consistently, 2- to 5-fold higher drug concentrations were required to kill fibroblasts compared with melanoma cells (Fig. 2B). Thus, cultured cancer cells are 2- to 5-fold more sensitive to PBISe than normal cells.

PBISe Inhibits Cellular Proliferation, Induces Apoptosis, and Arrests Cells in the G_2-M Phase of the Cell Cycle

To determine the mechanism by which PBISe inhibited cultured cancer cells, rates of cellular proliferation and apoptosis were examined in WM115, UACC 903, and 1205 Lu melanoma cells exposed to PBISe or PBIT. PBISe more effectively decreased cellular proliferation and increased apoptosis than PBIT in all three cell lines. Figure 2C and D are representative graphs illustrating changes in proliferation and apoptosis in UACC 903 cells exposed to PBISe or PBIT. Similar results were seen for WM115 and 1205 Lu cells (data not shown). Cell cycle analysis following PBISe but not PBIT exposure showed an increased sub–G_0-G_1 population (Fig. 3A), indicating elevated apoptosis and a ~3-fold increase in G_2-M cells (Fig. 3B and C), indicating a cell cycle arrest mediated by PBISe but not PBIT. Thus, PBISe inhibited proliferation by arresting cultured cells in the G_2-M phase of the cell cycle and induced apoptosis.

PBISe Modulates PI3K and MAPK Signaling Pathways Regulating Melanoma Development

Because deregulated PI3K (through overexpressed Akt3 and PTEN loss) and MAPK (through mutant ^{V600E}B-Raf) signaling promotes melanoma development (26, 27, 38), the effect of PBISe on these signaling cascades was examined by Western blot analysis of cellular lysates. Compared with PBIT, PBISe treatment inhibited Akt phosphorylation and decreased total Akt protein levels (Fig. 4A). Furthermore, phosphorylation of downstream Akt3 substrate PRAS40 was significantly inhibited with associated increases in cleaved caspase-3 and PARP indicating elevated apoptosis (Fig. 4A; ref. 29).

UACC 903, WM115, and 1205 Lu cells have elevated MAPK pathway signaling due to the presence of constitutively active ^{V600E}B-Raf (38, 39). Both PBIT and PBISe led to increased pErk1/2 in these cell lines, indicating increased MAPK pathway activity following treatment, which can be inhibitory to melanoma cells if the activity of this pathway

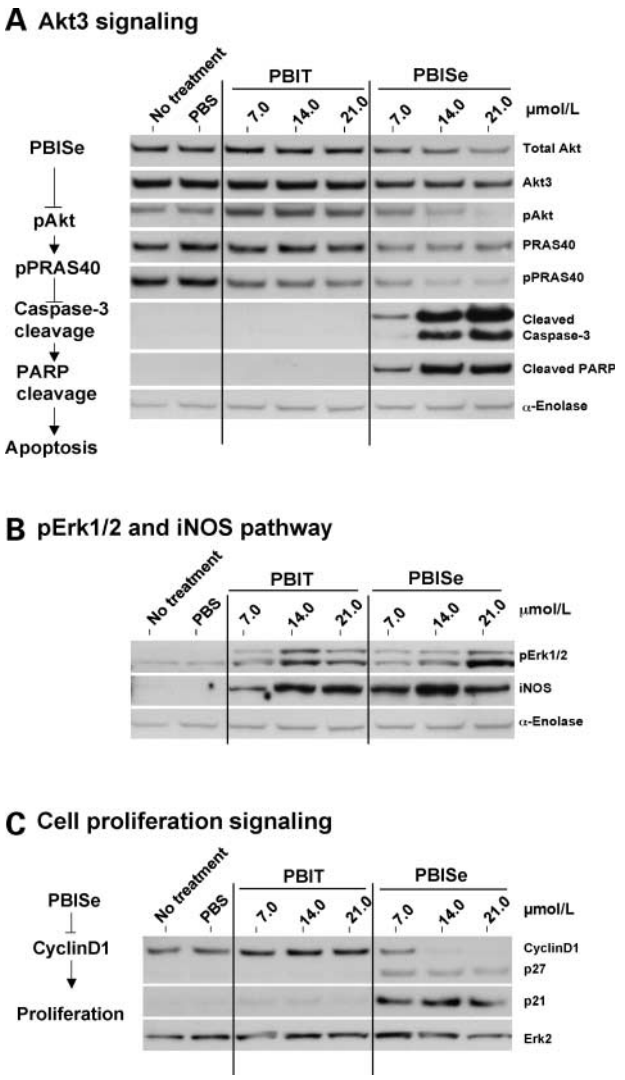


Figure 4. PBISe inhibits Akt3 signaling and activates MAPK activity in melanoma cells. **A,** PBISe inhibits Akt3 signaling in melanoma cell line UACC 903. UACC 903 cells (1×10^6) were treated with increasing concentrations (7 – 21 μmol/L) of PBIT and PBISe for 24 h and cell lysates examined by Western blot analysis for expression/activity of the Akt3 signaling pathway. PBISe decreased pAkt (S473) and pPRAS40 (T246) levels in a dose-dependent manner, causing significant apoptosis as indicated by elevated cleaved caspase-3 and PARP protein levels. Expression of total Akt was decreased compared with no treatment, PBS vehicle, or PBIT-treated cells. α-Enolase served as a control for equal protein loading. **B,** PBIT and PBISe increase MAPK pathway activity leading to elevated expression of downstream iNOS in melanoma cells. MAPK pathway activity and iNOS expression was measured in UACC 903 cells following treatment with increasing concentrations (7 – 21 μmol/L) of PBIT and PBISe for 24 h, and cell lysates were examined by Western blot analysis. Levels of pErk1/2 increased following both PBIT and PBISe treatments in a dose-dependent manner. Expression of iNOS also increased with elevated pErk1/2 levels consistent with prior reports (34). α-Enolase served as a control for protein loading. **C,** excessively high MAPK activation following PBISe treatment decreased cyclin D1 levels and increased p27 as well as p21 levels. Western blotting of UACC 903 cells treated with increasing concentrations (7 – 21 μmol/L) of PBIT and PBISe for 24 h showed that PBISe significantly reduced the proliferation marker cyclin D1 and increased the levels of cell cycle inhibitors p21 and p27 in UACC 903 melanoma cells. Erk2 served as a control for protein loading.

is overly active (representative example, Fig. 4B; ref. 36). In agreement with previous reports, increased pErk1/2 following PBIT treatment elevates iNOS expression (Fig. 4B; refs. 33, 34). However, PBIT did not alter cyclin D1 levels (Fig. 4C). In contrast, PBISe dramatically reduced the expression of cyclin-D1 and increased p27 as well as p21 levels in three cell lines (representative example, Fig. 4C). It is possible that this difference is due to PBISe-mediated inhibition of Akt3 activity, which can increase MAPK activity in melanoma cells by decreasing the phosphorylation of ^{V600E}B-Raf, which causes excessively high MAPK pathway activity (36). Under these circumstances, PBISe inhibits Akt3 activity making it unable to phosphorylate ^{V600E}B-Raf at Ser^{364} and Ser^{428} to reduce the activity of ^{V600E}B-Raf and downstream MAPK pathway to levels that promote rather than inhibit proliferation (36). The result is elevated MAPK activity reaching inhibitory levels, which decrease cyclin-D1 expression and increase p27 as well as p21 compared to controls (36). Thus, PBISe-mediated inhibition of Akt3 pathway signaling, increased MAPK activity to levels previously reported to be inhibitory, thereby increasing cellular apoptosis and decreasing cellular proliferation.

PBISe Reduces the Tumorigenic Potential of Melanoma Cells

The effectiveness of PBISe for inhibiting melanoma tumor development was evaluated on preexisting tumors in nude mice. 1205 Lu or UACC 903 human melanoma cells were injected s.c., and after 6 days, when a fully vascularized tumor had developed, mice were exposed to PBISe, PBIT (0.315 μmol/L each), or DMSO vehicle. Treatment with PBISe but not PBIT significantly reduced 1205 Lu (Fig. 5A) and UACC 903 (Fig. 5B) tumor development by ~30% to 50%. Thus, PBISe has the potential to appreciably reduce melanomas compared with PBIT.

PBISe Causes Negligible Organ-Related Toxicity following Systemic Administration

The systemic toxicity associated with PBISe in animals was evaluated in nude mice. Mice were exposed to PBISe or PBIT (0.315 μmol/L each) or vehicle DMSO, thrice a week, and animals were weighed to ascertain possible toxicity. No significant difference in body weight between PBISe-treated animals and controls was observed, suggesting negligible toxicity (see *insets* in Fig. 5A and B). Furthermore, blood parameters (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, blood urea, glucose, and creatinine) indicative of systemic toxicity did not detect significant liver, kidney, or cardiac-related toxicity (Fig. 5C). Histologic examination of H&E-stained vital organ sections revealed that PBISe treatment did not significantly change cell morphology or organ structure (data not shown). Thus, PBISe treatment has negligible associated systemic toxicity at the concentrations examined.

PBISe Induces Cellular Apoptosis in the Tumor Environment

Because PBISe significantly reduced tumor development, subsequent studies focused on identifying the mechanism by which inhibition occurred. Rates of tumor cell apoptosis

and proliferation were compared in size-matched and time-matched tumors following PBISe, PBIT, or control DMSO vehicle treatment. The magnitude of apoptosis, assessed by caspase-3/7 activities in tumor protein lysates, were examined (Fig. 5D). UACC 903 tumors treated with PBISe had double the caspase-3/7 activity by day 15 observed in tumors treated with PBIT or DMSO vehicle (Fig. 5D, $P < 0.05$; one-way ANOVA). In contrast, no statistically significant difference was observed in rates of cellular proliferation, which remained between 1% and 2% for PBISe-treated and PBIT-treated tumors (data not shown). However, PBISe-treated and PBIT-treated tumors had 2- to 3-fold fewer proliferating cells than animals exposed to control DMSO. Thus, PBISe inhibited melanoma tumor development by increasing apoptosis levels compared with PBIT but no significant differences in proliferation rates were observed between the two compounds.

Discussion

Melanoma is one of the most difficult cancers to treat in its advanced stages (4, 26, 40). Cells are resistant to most untargeted chemotherapeutic agents, which are driving

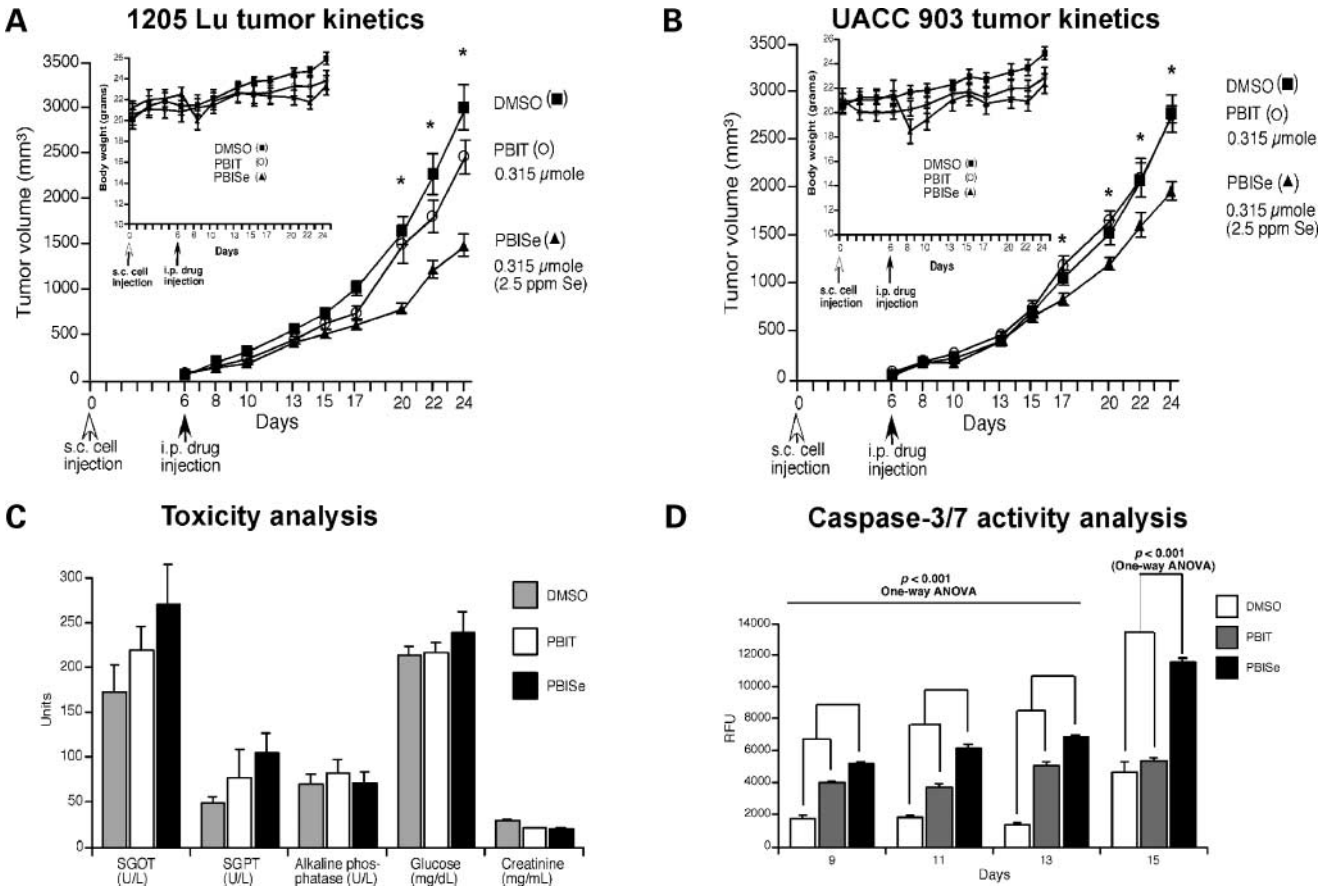


Figure 5. PBISe treatment decreased melanoma development. PBISe decreases the tumorigenic potential of melanoma 1205 Lu cells (**A**) by ~50% and UACC 903 by ~30% (**B**), with a negligible effect on animal body weight. UACC 903 (5×10^6) and 1205 Lu (2.5×10^6) melanoma cells were injected s.c. into left and right flanks near the rib cages of 4- to 6-week-old female nude mice and tumors allowed to develop for 6 d, at which time, a fully vascularized tumor had developed. From day 6, mice were injected i.p. with PBIT (0.315 μmol/L), PBISe (0.315 μmol/L, 2.5 ppm of selenium), or DMSO vehicle every Monday, Wednesday, and Friday for 3 wks. The sizes of developing tumors and body weights (*inset*) were measured at the time of each drug injection. *Points,* average values obtained from 10 tumors ($n = 5$ animals); *bars,* SE. Compared with PBIT or DMSO vehicle, PBISe significantly decreased tumor development beginning from day 17 ($P < 0.001$; two-way ANOVA). At day 24, the difference between control DMSO or PBIT compared with PBISe-treated tumor volumes was ~50% for 1205 Lu cells (**A**) and ~30% for UACC 903 cells (**B**). No significant difference was observed in body weight during treatment indicating negligible toxicity. **C,** blood markers for major organ–related toxicity indicate negligible toxicity at the concentrations examined. Levels of aspartate aminotransferase (for serum glutamic oxaloacetic transaminase), alanine aminotransferase (for serum glutamic pyruvic transaminase), alkaline phosphatase, glucose, and creatinine were analyzed in blood collected from the animals treated with PBIT, PBISe, or DMSO vehicle. Treatment with PBIT or PBISe did not significantly alter any of these variables compared with vehicle DMSO–treated animals, indicating negligible vital organ–related (liver, heart, kidney, and pancreas) toxicity. **D,** PBISe treatment promotes apoptosis in size-matched and time-matched melanoma tumors. Mice bearing tumors matched for size and time of development were injected i.p. with PBIT and PBISe (0.315 μmol/L) or DMSO (50 μL) vehicle starting 7 d after s.c. injection of cells and on alternate days thereafter up to day 15. Tumors were removed from euthanized mice on days 9, 11, 13, and 15 for measurement of caspase-3/7 activity using the Apo-ONE homogeneous caspase-3/7 assay kit. Progressively, statistically higher levels of apoptosis (caspase-3/7 activity) were observed from tumors at days 9 to 15, with a 2-fold difference measured at day 15. *Columns,* average from three independent experiments; *bars,* SE.

the search for agent combinations targeting proteins or signaling cascades deregulated during development (4, 5). For the targeted approach to be effective, deregulated proteins or signaling pathways need to be identified and potent drugs developed inhibiting these targets, which are effective in preclinical as well as clinical systems (26, 40).

PBISe is more effective than PBIT at killing melanoma cells because it inhibits proliferation and survival pathways that have been shown to be important for melanoma development. The PI3K and MAPK pathways play prominent roles in melanoma by relaying extracellular signals from cell surface to nucleus, thereby regulating diverse cellular processes including cellular proliferation and survival (41). Increased activity of the PI3K pathway occurs in ~70% of sporadic melanomas due to loss of PTEN and/or increased expression of Akt3 resulting from an increase in the gene copy number (27, 28). The highest level of Akt3 activity occurs in metastatic melanomas, deregulating apoptotic signaling and promoting chemoresistance (27). In this study, PBISe is shown to inhibit Akt3 signaling leading to increased levels of melanoma cell apoptosis in cultured cells and melanoma tumors mediated by caspase-3/7 and PARP cleavage.

PBISe also regulates the activity of the MAPK pathway, which is activated through Ras mutations in 10% to 15% of melanomas and B-Raf mutations in ~60% of melanomas (32, 42–44). Moderate levels of MAPK pathway activation promote cell cycle progression and cancer development whereas excessively high levels inhibit cellular growth in a wide variety of normal and cancer cells, including melanomas, by promoting cellular senescence (36, 45). Senescence is linked to the induction of a variety of cyclin dependent kinase inhibitors, such as $p21^{Cip1}$, $p16^{Ink4a}$, and $p27^{Kip1}$, which functions as a putative defense mechanism of normal cells to overcome oncogene activation (46–48). PBIT has been reported to increase MAPK activity in cells, which is confirmed to occur in this study following either PBIT or PBISe treatment (34, 36).

This study shows the preclinical evaluation of a novel selenium-containing small molecule inhibitor developed from the well-characterized iNOS inhibitor PBIT (13). PBIT is a non–amino acid competitive inhibitor of iNOS (6, 13) which, this study shows, is ineffective at killing melanoma cells. However, the selenium-containing analog, PBISe, has improved chemotherapeutic inhibitory activities making it >10-fold more effective at killing melanoma cells compared with PBIT. In addition to targeting iNOS similar to PBIT, PBISe also acts as a PI3K signaling cascade inhibitor in melanoma cells, making Akt3 unable to phosphorylate ^{V600E}B-Raf at Ser^{364} and Ser^{428}. Akt3 has been shown in melanomas to directly regulate the activity of mutant ^{V600E}B-Raf by phosphorylating Ser^{364} and Ser^{428} to reduce the activity of ^{V600E}B-Raf and the downstream MAPK pathway (36). Because PBISe inhibits Akt3 activity, Akt3 is unable to phosphorylate ^{V600E}B-Raf, which elevates MAPK activity to levels that inhibit rather than promote melanoma development (36). Excessively elevated MAPK activity following PBISe treatment then causes a G_2-M cell cycle block mediated by decreased cyclin D1 and increased p21 as well as p27 levels (36). This novel property of PBISe causes decreased cell proliferation and increased apoptosis of melanoma cells. Furthermore, PBISe kills cultured cancer cells at concentrations that are 2- to 5-fold less than would be required for killing normal cells. Thus, PBISe has significant potential as a melanoma therapeutic.

By targeting the PI3K and MAPK pathways in melanomas, PBISe effectively reduces the growth of established tumors by 30% to 50% with negligible associated systemic toxicity. Caspase-3/7 activity in size-matched and time-matched tumors was increased 2-fold in PBISe-treated animals compared with controls. Similarly, studies targeting Akt3 in melanomas using either small interfering RNA or small molecule inhibitors showed that decreased Akt3 activity reduced tumor volume by increasing apoptosis (27, 29). PBISe-treated and PBIT-treated tumors also had 2- to 3-fold fewer proliferating cells than animals exposed to control DMSO. Because Akt3 is a key protein deregulated in melanoma, inhibitors such as PBISe that reduce its activity will be a key part of an effective chemotherapeutic cocktail of agents for successfully treating melanoma.

Several reports have documented the beneficiary effects associated with selenium for preventing cancer development (17, 49). Dietary consumption of selenium-enriched cruciferous vegetables such as broccoli, cabbage, and cauliflower has been shown to reduce the incidence of esophageal, colorectal, lung, and prostate cancers (17, 50). Synthetic and naturally occurring organoselenium compounds such as p-XSC, methyl seleninic acid, methyl selenol, and selenocysteine significantly decrease melanoma metastasis and lung cancer development (20, 21, 24). Although the exact mechanism by which selenium prevents cancer development remains uncertain, selenium is present as a constituent of proteins, modifies the activities of various enzymes, and regulates protein transport (49, 51). It also maintains the cellular redox balance thereby protecting cell membranes and lipid messengers within cells (50). Several studies have shown that serum from patients with advanced-stage melanoma contains significantly lower levels of selenium compared with serum from control patients; and that dietary supplementation can improve the quality and duration of life (18, 22). Thus, selenium plays an important role in preventing and treating cancer. Therefore, incorporating selenium into the structure of compounds could lead to the creation of more effective therapeutic agents as shown through the development of PBISe from PBIT.

In conclusion, selenium-containing PBISe has been shown to be a more effective therapeutic agent than PBIT. It kills melanoma cells >10-fold more effectively than PBIT and cultured cancer cells are 2- to 5-fold more sensitive than normal cells. Compared with PBIT, PBISe decreases the growth of established melanomas by 30% to 50% inducing a 2-fold increase in apoptosis and 2- to 3-fold decrease in tumor cell proliferation with negligible associated systemic toxicity. Thus, PBISe is a potent chemotherapeutic agent with novel properties enabling the targeting

of iNOS, Akt3, and MAPK signaling, thereby promoting melanoma cell apoptosis while inhibiting proliferation. To achieve complete melanoma tumor remission, future studies could combine PBISe with agents that would induce cellular apoptosis because decreasing Akt3 activity mediated by this compound would sensitize the cells to killing via these agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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