Targeting Akt3 Signaling in Malignant Melanoma Using Isoselenocyanates

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Abstract

Purpose: Melanoma is the most invasive and deadly form of skin cancer. Few agents are available for treating advanced disease to enable long-term patient survival, which is driving the search for new compounds inhibiting deregulated pathways causing melanoma. Akt3 is an important target in melanomas because its activity is increased in ~70% of tumors, decreasing apoptosis in order to promote tumorigenesis.

Experimental Design: Because naturally occurring products can be effective anticancer agents, a library was screened to identify Akt3 pathway inhibitors. Isothiocyanates were identified as candidates, but low potency requiring high concentrations for therapeutic efficacy made them unsuitable. Therefore, more potent analogs called isoselenocyanates were created using the isothiocyanate backbone but increasing the alkyl chain length and replacing sulfur with selenium. Efficacy was measured on cultured cells and tumors by quantifying proliferation, apoptosis, toxicity, and Akt3 pathway inhibition.

Results: Isoselenocyanates significantly decreased Akt3 signaling in cultured melanoma cells and tumors. Compounds having 4 to 6 carbon alkyl side chains with selenium substituted for sulfur, called ISC-4 and ISC-6, respectively, decreased tumor development by ~60% compared with the corresponding isothiocyanates, which had no effect. No changes in animal body weight or in blood parameters indicative of liver-, kidney-, or cardiac-related toxicity were observed with isoselenocyanates. Mechanistically, isoselenocyanates ISC-4 and ISC-6 decreased melanoma tumorigenesis by causing an ~3-fold increase in apoptosis.

Conclusions: Synthetic isoselenocyanates are therapeutically effective for inhibiting melanoma tumor development by targeting Akt3 signaling to increase apoptosis in melanoma cells with negligible associated systemic toxicity.

Dacarbazine is one of the approved chemotherapeutic agents for metastatic melanoma but is relatively ineffective with an overall response rates of 5% to 20% (1, 2). This is equally true of most currently available therapeutic strategies for metastatic melanoma patients, including surgery, immunotherapy, radiotherapy, and chemotherapy, which are ineffective long-term treatments for individuals suffering from advanced disease (3, 4). This is a serious problem because the incidence of melanoma remains unchecked, increasing at a rate of ~4% per year and is predicted to affect 1 in 50 US citizens by 2010. As a direct result of the lack of effective therapeutics, the current prognosis for patients with metastatic disease remains very poor, with average survival ranging from 6 to 10 months (2, 5).

To develop more effective melanoma therapeutics, agents targeting proteins promoting disease development are being developed (6–11). Akt3 is one example, which is activated in ~70% of melanomas (6–8). Although three Akt isoforms known as Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ (12, 13) are expressed in melanocytes and melanoma cells, Akt3 is the predominantly active family member (6). Increased *AKT3* gene copy number and/or loss of a negative regulatory phosphatase called PTEN leads to Akt3 activation (6, 14), which reduces melanoma cell apoptosis mediated through caspase-3 to promote melanoma development (6). Recently, PRAS40 was identified as an important downstream protein in the Akt3 signaling cascade regulating cellular apoptosis (7). Inhibiting PRAS40 or Akt3 using small interfering RNA (siRNA)-based approaches increased cellular apoptosis to similar levels, causing a significant reduction in the tumorigenic potential of

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Translational Relevance

This article is clinically relevant because melanoma is the most invasive and deadly form of skin cancer with few agents available to treat advanced metastatic disease. Thus, research scientists are vigorously searching for new therapeutic agents targeting pathways important in melanoma to treat the disease. One gene involved in ~70% of sporadic melanomas is *AKT3*, promoting tumorigenesis by decreasing apoptosis. Here we detail identification of naturally occurring isothiocyanates, present in cruciferous vegetables, as inhibitors of the Akt3 pathway in melanoma. However, low potency requiring high concentrations for therapeutic efficacy made them unsuitable therapeutics. Therefore, more potent analogs have been developed using the isothiocyanate backbone but increasing the alkyl chain length and replacing sulfur with selenium to create compounds called isoselenocyanates. Isoselenocyanates decreased Akt3 signaling in cultured melanoma cells and tumors to significantly reduce melanoma tumor development without changes in animal body weight or in blood parameters indicative of liver-, kidney-, or cardiac-related toxicity.

melanoma cells (7). A second function for Akt3 in early melanoma development has also been reported recently (15). Akt3 has been shown to phosphorylate a constitutively active mutant form of B-Raf, called ^{V600E}B-Raf, and in so doing, lowers the activity of the mutant protein to levels that promote rather than inhibit melanoma tumor progression (15). Therefore, targeting Akt3 would also have the added consequence of increasing ^{V600E}B-Raf activity to levels that would be inhibitory to growth. Although these studies show the therapeutic potential of targeting the Akt3 signaling cascade to inhibit melanoma development, no clinical agent is available for inhibition of Akt3 signaling in melanoma.

Isothiocyanates are naturally occurring compounds found in cruciferous vegetables having anticancer properties (16–19), protecting against murine tumorigenesis induced by environmental carcinogens such as polycyclic aromatic hydrocarbons and nitrosamines (20, 21). Certain studies suggest the mechanism of action of isothiocyanates is by inhibiting the PI3 kinase pathway (22, 23). Selenium is also an effective chemopreventive agent by modulating Akt activity (24–26). Selenium deficiency occurs frequently in cancer patients including those diagnosed with metastatic melanoma (27). Recently, selenium has been shown to induce destabilization of Akt activity in prostate cancer cells (28, 29). Therefore, incorporating selenium into the structure of compounds could increase compound efficacy and these compounds would be safe because melanoma patients frequently have selenium deficiency.

In this study, isothiocyanate analogs having longer carbon chain lengths and selenium substituted for sulfur were developed, and the therapeutic efficacy for killing cultured melanoma cells or inhibiting tumor development in animals was evaluated. Although increasing chain length did not increase the tumor inhibitory potency of sulfur-containing isothiocyanates, the incorporation of selenium with increasing chain length significantly enhanced antitumor potency by elevating rates of tumor cell apoptosis. Thus, novel isoselenocyanates have been developed that target Akt3 signaling in melanoma cells leading to robust antimelanoma activity.

Materials and Methods

Cell lines and culture conditions. The human fibroblast cells FF2441 and metastatic melanoma cell lines UACC 903 and 1205 Lu were maintained in Dulbecco's modified eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37°C with 5% CO_2 atmosphere in a humidified incubator. Vertical growth phase melanoma cell line WM115 was maintained in Tu2% medium as described previously (6).

Western blot analysis. For Western blot analysis, floating and adherent cells treated with compounds or control vehicle (DMSO) were harvested by addition of lyses buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mmol/L sodium molybdate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 5 μg/mL leupeptin. Whole cell lysates were centrifuged ($\geq$10,000 × *g*) for 10 min at 4°C to remove cell debris. Protein concentrations were quantitated using the BCA assay from Pierce, and 30 μg of lysate were loaded per lane onto NuPAGE Gels from Life Technologies. Following electrophoresis, samples were transferred to a polyvinylidene difluoride membrane (Pall Corporation). Blots were probed with antibodies according to each supplier's recommendations: phosphorylated-PRAS40 (Thr246) from Invitrogen; phosphorylated Akt (Ser473), Akt3, and cleaved PARP from cell signaling; Erk2, α-enolase, and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology; and Immunoblots were developed using the enhanced chemiluminescence detection system (Pierce Biotechnology).

Synthesis of isothiocyanates, isoselenocyanates, and phenylhexyl selenocyanate. The isothiocyanates (ITC) BITC and PEITC were purchased from Sigma-Aldrich. PBITC and PHITC were synthesized by using previously reported methodology (30). Isoselenocyanates (ISC) were synthesized using a described method.[7] Briefly, a solution of triphosgene (5 mmol) in CH_2Cl_2 (15 mL) was added over a refluxing mixture of formamides (10 mmol), triethylamine (43 mmol) and 4 Å molecular sieves in CH_2Cl_2 (35 mL). The mixture was then refluxed for an additional 2.5 h. Selenium powder (20 mmol) was then added and the resulting mixture refluxed for 6 to 8 h. The mixture was cooled, filtered, and solvent evaporated yielding a crude mixture, which was purified by silica gel column chromatography generating pure isoselenocyanates. 6-phenylhexylselenocyanate (PHSC) was prepared by reacting 0.3 g (1.25 mmol) of 6-phenylhexylbromide with 0.19 g (1.32 mmol) of KSeCN in 10 mL of acetonitrile, in a nitrogen atmosphere. After stirring overnight at room temperature, the residue was partitioned between ethyl acetate and water. The organic phase was separated, washed with brine and water, dried over $MgSO_4$, filtered, and the solvent evaporated to yield 0.26 g (74% yield). The compounds' identities were confirmed by nuclear magnetic resonance as well as mass spectra analysis, and purity (>99%) was quantified by high-performance liquid chromatography analysis.

SiRNA protein knockdown studies. Duplexed "Stealth" siRNA from Invitrogen were: AKT3-5′-GGA CUA UCU ACA UUC CGG AAA GAU U-3′ and scrambled-5′-AAU UCU CCG AAC GUG UCA CGU GAG A-3′. Nucleofection using Amaxa Nucleofector was used to introduce siRNA into UACC 903 cells (Reagent R, program K17). SiRNA (100 pmoles) against Akt3 or scrambled siRNA or buffer were nucleofected into 1 ×

[7] Sharma AK, Sharma A, Desai D, Madhunapantula SV, Huh SJ, Robertson GP, Amin S. Synthesis and anticancer activity comparison of isoselenocyanates with isothiocyanates present in cruciferous vegetables. Submitted.

10^6 UACC 903 cells, which were then replated in DMEM supplemented with 10% FBS and allowed to recover for 36 h. Transfection efficiency was >90% with ~80% viability. For animal experiments, 36 h later, 1×10^6 viable UACC 903 cells in 0.2 mL of DMEM supplemented with 10% FBS were injected s.c. into the left and right flanks of 3- to-4-wk-old female Athymic Nude-Foxn1^{nu} mice. The dimensions of developing tumors were measured on alternate days using calipers up to day 17.5. To test duration of siRNA-mediated knockdown, protein lysates were collected at 2, 4, 6, and 8 d following nucleofection and measured for Akt3 protein expression by Western blot analysis and quantitated by densitometry as described previously (6, 14).

Cell viability, proliferation, apoptosis determination and cell cycle analysis. Viability and IC_{50} of melanoma cells following treatment with compounds was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). Briefly, 5×10^3 melanoma (UACC 903, 1205 Lu, or WM115) or human fibroblast (FF2441) cells per well in 100 μL DMEM containing 10% FBS were grown in a 96-well plate for 24 or 76 h and treated with either control DMSO vehicle or increasing concentrations (2-100 μmol/L) of compounds for 24 h. Cellular viability compared with vehicle control–treated cells was measured using the MTS assay. IC_{50} values for each compound in respective cell lines was determined from three independent experiments using GraphPad Prism version 4.01 (GraphPad software).

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

Cell cycle analysis was undertaken by plating 1.5×10^6 melanoma cells in a 100-mm culture dish and following treatment with respective compounds for 24 h; total cells (floating and adherent) were trypsinized, centrifuged ($500 \times g$, for 5 min), and treated with 1 mL of propidium iodide staining solution containing 100 μg/mL propisium iodide (Sigma), 20 μg/mL Ribonuclease A (Roche Applied Sciences), 3 μg/mL Triton X-100 dissolved in 0.1% (w/v) sodium citrate for 30 min at 4°C (31). Cells were analyzed using the FACScan analyzer (Becton Dickinson) and data processed using ModFit LT software (Verity software house).

Tumorigenicity assessment, knockdown of protein expression and measurement of proliferation/apoptosis rates in tumors. Animal experimentation was done according to protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University College of Medicine. Tumor kinetics were measured by s.c. injection of $2.5\text{-}5 \times 10^6$ 1205 Lu or UACC 903 melanoma cells in 0.2 mL of DMEM supplemented with 10% FBS above both left and right rib cages of 3- to 4-wk-old female Athymic Nude-Foxn1^{nu} mice (Harlan Sprague Dawley). Six days later, when a fully vascularized tumor (50-75 mm^3) had formed, the mice were randomly divided into DMSO vehicle control and experimental (BITC, PEITC, PBITC, PHITC, ISC-1, ISC-2, ISC-4 or ISC-6) groups (5 mice/group; 2 tumors/mouse) and treated i. p. with isothiocyanate or isoselenocyanate compounds [2.5 or 0.76 μmoles (equivalent to 3 ppm selenium)/20 g mice in 50 μL DMSO vehicle] on Monday, Wednesday, and Friday for ~ 3 wk. Control mice received an equivalent volume of the vehicle. Body weight (grams) and dimensions of the developing tumors (mm^3) were measured at the time of treatment.

To ascertain the mechanism underlying tumor inhibition, 5×10^6 UACC 903 cells were injected into nude mice; 6 d later the mice were treated i. p. with PBITC or PHITC (0.76 μmoles), ISC-4 or ISC-6 (0.76 μmoles, equivalent to 3 ppm selenium) on alternate days. Size- and time-matched tumors were harvested on days 11 and 13 to assess changes in cell proliferation and apoptosis. A small portion of the tumor was also flash-frozen in liquid nitrogen, pulverized, and lysed in protein lysis buffer (600-800 μL, 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 pyrophosphate, 1 mmol/L activated sodium orthovanadate, protease inhibitor cocktail from Sigma, and 0.1%, v/v, 2-mercaptoethanol). Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad laboratories), analyzed by Western blotting to measure levels of pAkt and downstream pPRAS40 in tumors, and quantitated by densitometry as described previously (6, 14).

Cell proliferation and apoptosis were measured in formalin-fixed, paraffin-embedded tumor sections using the TUNEL TMR Red Apoptosis kit from Roche or purified mouse antihuman Ki-67 from PharMingen, respectively. A minimum of six different tumors with four to six fields per tumor were analyzed and results represented as the average ± SE.

Toxicity assessments. Four- to six-week-old female nude mice were injected i. p. with either control DMSO vehicle, PBITC or PHITC (0.76 μmoles) or ISC-4 or ISC-6 (0.76 μmoles equivalent to 3 ppm Se) on Monday, Wednesday, and Friday for 3 wk. Animals were sacrificed by CO_2 asphyxiation and blood was collected from each animal in plasma separator tubes with lithium heparin (BD) following cardiac puncture and analyzed for serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, 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), 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 liver, heart, kidney, spleen, intestine, pancreas, and adrenal from each animal was formalin-fixed and paraffin-embedded to examine toxicity-related changes in cell or organ morphology by H&E staining.

Statistical analysis. Statistical analysis was carried out using Prism 4.0 (GraphPad Software). One-way or two-way ANOVA was used for groupwise comparisons, followed by the Tukey's or Bonferroni's post hoc tests. All the data represented as ± SE. Results were considered significant at $P < 0.05$ (95% confidence interval).

Results

siRNA-mediated inhibition of Akt3 signaling reduced the tumorigenic potential of melanoma cells. To confirm prior studies documenting the therapeutic potential of inhibiting Akt3 signaling in melanoma tumorigenesis, a siRNA-based approach was initially used to inhibit protein expression and thereby activity (6, 7, 15). UACC 903 cells were nucleofected with siRNA targeting Akt3, or a scrambled siRNA or a buffer control using the Amaxa nucleofection system. Thirty-six hours later, viable cells were s.c. injected into nude mice and tumor development was measured every other day. Decreased expression (activity) of Akt3 reduced the tumorigenic potential of melanoma cells by ~60% (one-way ANOVA, $P < 0.001$) compared with control cells nucleofected with scrambled siRNA or nucleofection buffer (Fig. 1A). A tumor removed from animals on day 9.5 showed significantly less Akt3 protein than control (scrambled siRNA) tumors showing effective knockdown of Akt3 protein expression (Fig. 1B). Duration of Akt3 protein knockdown following exposure to siRNA targeting Akt3 persisted up to 8 days in culture (Fig. 1C) as reported previously (6). Thus, targeting Akt3 signaling led to significant melanoma tumor inhibition, which has laid the foundation to search for pharmacologic agents that could inhibit melanoma development by reducing the activity of this important signaling cascade involved in ~70% of sporadic melanomas (6).

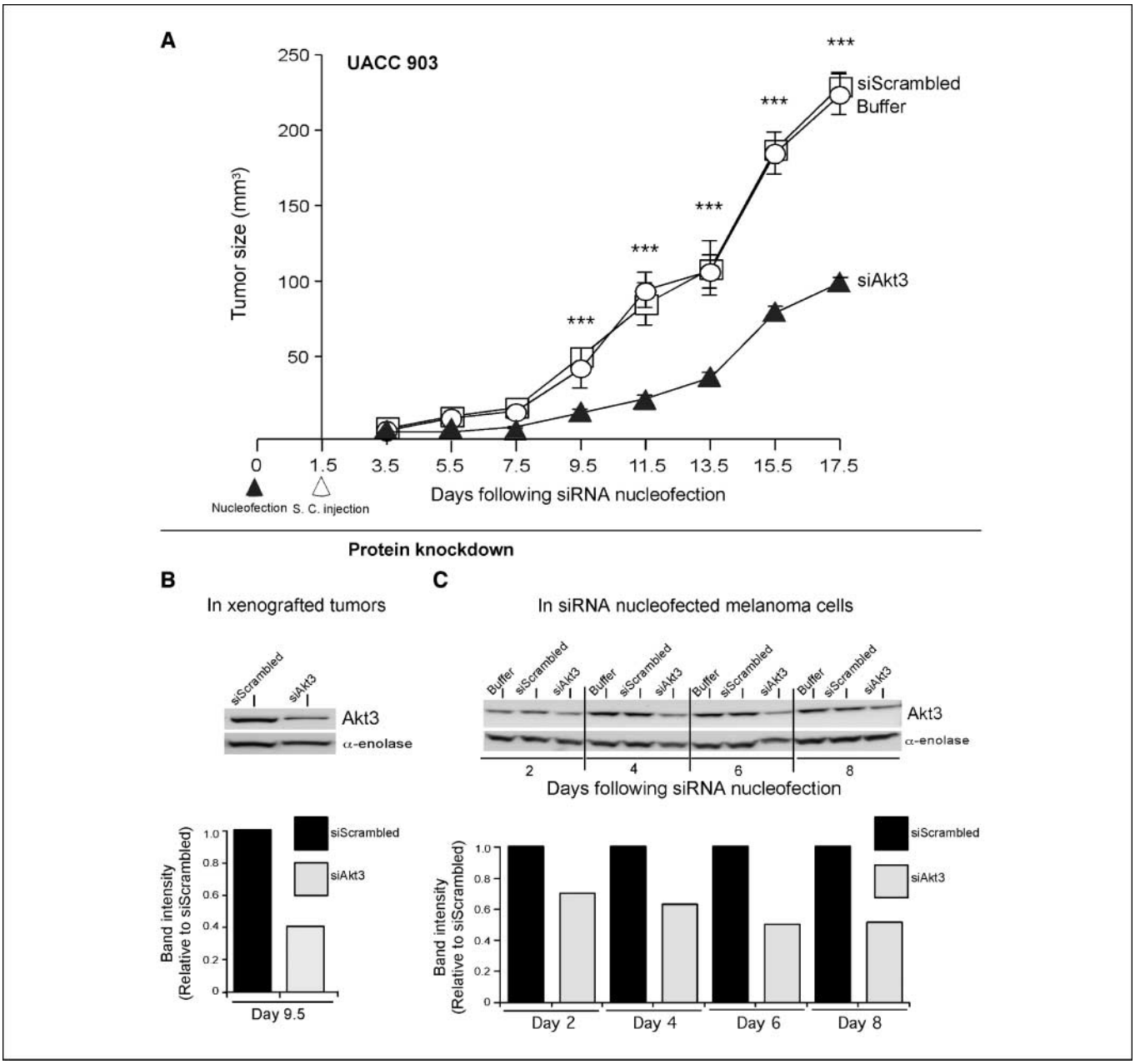


Fig. 1. siRNA-mediated inhibition of Akt3 expression/activity inhibits melanoma tumor development. *A,* siRNA (100 pmoles) against Akt3 (▲), scrambled siRNA (□), or nucleofection buffer (○) were introduced into UACC 903 (1×10^6) melanoma cells and 36 h later viable cells were injected s.c. into nude mice. Result shows that reduced expression/activity of Akt3 decreases the tumorigenic potential of melanoma cells by ~60% compared with control cells nucleofected with scrambled siRNA or nucleofection buffer (one-way ANOVA, $P < 0.0001$); error bars, SE. *B,* Western blot and quantitation analysis of tumor protein lysates harvested 9.5 d after cells were nucleofected and s.c. injected into animals. Decreased Akt3 protein expression was observed in tumors confirming siRNA-mediated inhibition. α-Enolase served as a control for protein loading. *C,* siRNA-mediated knockdown of Akt3 protein persists up to 8 d in cultured cells. One million UACC 903 cells were nucleofected with 100 pmoles of siAkt3 and replated in culture dishes, and protein lysates harvested 2, 4, 6, and 8 d later for Western blot analysis to determine Akt3 protein levels in cells. Decreased Akt3 protein expression compared with controls was observed up to 8 d after nucleofection into cells. α-Enolase served as a control for protein loading on Western blots, whereas scrambled siRNA served as a control for RNA interference specificity.

Development of isothiocyanate analogs with longer alkyl chain lengths and selenium substituted for sulfur. In order to identify a pharmacologic agent inhibiting Akt3 activity and melanoma cell survival, a natural product library was screened and isothiocyanates identified as possible candidates. The parent isothiocyanate compound, however, had low potency *in vivo* requiring high concentrations for efficacy. Therefore, more potent analogs were created that could serve as therapeutic agents using the isothiocyanate backbone. The goal was to identify optimal carbon chain length for maximal tumor inhibition by comparing arylalkyl isothiocyanate compounds with increasing alkyl chain length and by replacing sulfur with selenium. Figure 2A shows the structures of isothiocyanates containing 1(benzyl), 2 (phenethyl), 4 (phenylbutyl), and 6 (phenylhexyl) carbon spacers. Corresponding isosteric selenium analogs are also shown in which sulfur was replaced

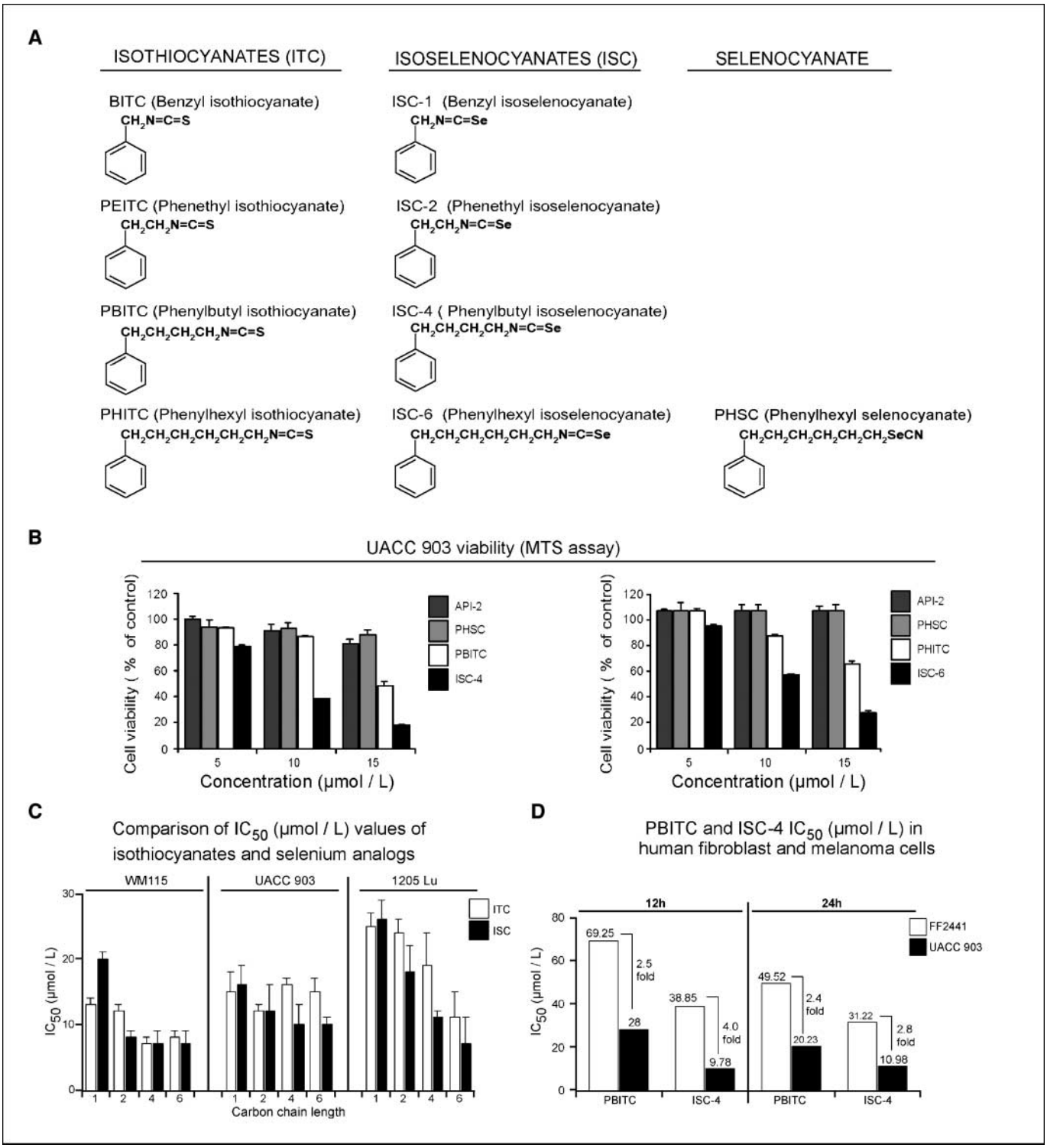


Fig. 2. Characterization of isothiocyanates and isoselenocyanates as inhibitors of melanoma. *A,* structures of isothiocyanates and selenium-containing isoselenocyanates. Chemical structures of isothiocyanates containing 1 (benzyl), 2 (phenethyl), 4 (phenylbutyl), and 6 (phenylhexyl) carbon spacers and corresponding isosteric selenium analogs in which sulfur was replaced with selenium. A 6-carbon selenocyanate, PHSC, served as a control to show compound structure and not selenium caused the inhibitory effect. *B* and *C,* comparison of melanoma cell survival following exposure to isothiocyanates versus isoselenocyanates. Cell viability was measured using the MTS assay and IC_{50} (μmol/L) values plotted against carbon chain length. 5×10^3 melanoma cells were plated in 96-well plates and allowed to attach for 24 h. Increasing concentrations of isothiocyanates and selenium analogs were added in culture medium. Values represent averages of percentage of control DMSO-treated cells. API-2 and PHSC served as an Akt inhibitor and selenium control, respectively. Results show that ISC-4 and ISC-6 were the most effective inhibitors. *D,* ISC-4 kills melanoma cells at 2- to 4-fold lower concentrations than normal cells. 5×10^3 normal human fibroblasts (FF2441) or metastatic melanoma cells (UACC 903) were plated in 96-well plates in 100 μL DMEM media containing 10% FBS and grown for 72 and 36 h, respectively. Exponentially growing cells were treated with increasing concentrations (2-100 μmol/L) of ISC-4 or PBITC for 12 and 24 h, and IC_{50} (μmol/L) values were determined. ISC-4 was found to consistently inhibit melanoma cells growth at concentrations 2- to 4-fold lower than fibroblast cells (one-way ANOVA, $^{***}P < 0.001$); error bars, SE.

with selenium. Finally, a 6 carbon selenocyanate, PHSC, was created to serve as a control to show that selenium alone was not accounting for inhibition but rather that the structure of the compound containing selenium was critical for tumor reduction (Fig. 2A).

Isoselenocyanates are more effective at inhibiting cultured melanoma cells than isothiocyanates. Initially, the MTS assay was used to quantify viable cells of three human melanoma cell lines (UACC 903, 1205 Lu, and WM115) following treatment with increasing concentrations of each agent to measure the IC_{50} of the respective compounds. Figure 2B shows a representative example of this analysis where the inhibitory effectiveness of the 4 carbon PBITC and ISC-4 as well as 6 carbon PHITC and ISC-6 were compared with DMSO vehicle, Akt inhibitor API-2 (1,5-Dihydro-5-methyl-1-b-D-ribofuranosyl-1,4,5,6,8-penta azaacenaphthylen-3-amine; refs. 7, 32), or control PHSC. Selenium-containing ISC-4 and ISC-6 were more effective at inhibiting the growth of melanoma cells than sulfur-containing PBITC, PHITC, control PHSC, or API-2. Figure 2C shows a detailed comparison where the IC_{50} of 1, 2, 4, or 6 carbon isothiocyanates are compared with selenium-containing analogs in three independently derived melanoma cell lines, UACC 903, 1205 Lu, and WM115. A general trend was observed in which increasing carbon chain length and substitution of selenium for sulfur decreased the IC_{50} for all cell lines but the differences were subtle. Increased potency ranged from 30% to 70% with increasing chain length and/or sulfur substituted for selenium. Thus, isothiocyanate analogs with longer alkyl chain lengths and sulfur substituted for selenium had increased killing efficiency for cultured melanoma cells.

Isoselenocyanates inhibits melanoma cell growth more effectively than normal cells. Sensitivity of melanoma and normal cells to PBITC or ISC-4 was compared to determine whether cancer cells were more sensitive to the compounds. Normal human fibroblast, FF2441, and melanoma (UACC 903) cells were treated with 2 to 100 μmol/L of PBITC or ISC-4, and IC_{50} measured at 12 and 24 hours (Fig. 2D). Consistently, 2- to 4-fold higher drug concentrations were required to kill fibroblasts compared with melanoma cells (Fig. 2D). Thus, cultured cancer cells are more sensitive to PBITC or ISC-4 than are normal cells.

Isoselenocyanates have increased* in vivo *potency compared with corresponding isothiocyanates and effectively reduce melanoma development. The effectiveness of isoselenocyanates for inhibiting the growth of preexisting tumors was evaluated in

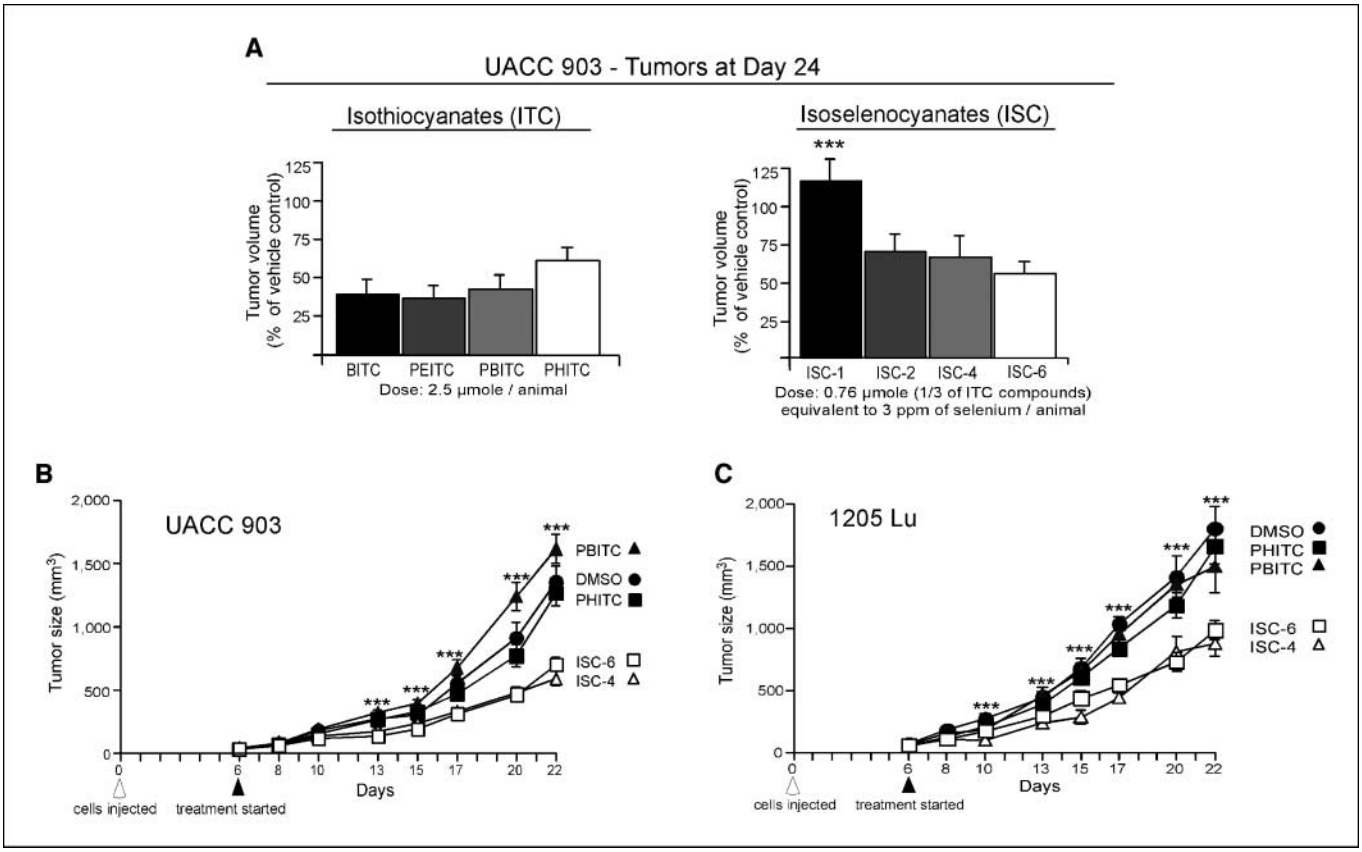


Fig. 3. Isoselenocyanates are effective inhibitors of melanoma tumor development. *A,* Isoselenocyanates inhibit melanoma development at concentrations 3-fold less than corresponding isothiocyanates. Effect of isothiocyanates and isoselenocyanates on melanoma tumor development was measured by s.c. injection of 5 million UACC 903 cells and after 6 d when small vascularized palpable tumors were observed, mice were treated i. p. with isothiocyanates (2.5 μmoles) or isoselenocyanates (0.76 μmoles, equivalent to 3 ppm selenium) 3 times per week. Bar graph, means of melanoma tumor volume (n = 10) as the percentage of vehicle-treated tumors at d 24; error bars, SE. Results show that similar tumor inhibition required 3-fold less isoselenocyanate than isothiocyante compound. *B* and *C,* isoselenocyanates decrease melanoma tumor development by 50% to 60% compared with corresponding isothiocyanates at similar concentrations. Effect of isoselenocyanates on tumor development was measured by s.c. injection of 2.5 or 5 million 1205 Lu or UACC 903 melanoma cells, respectively, and after 6 d, mice were treated i. p. 3 times per week with ISC-4 or ISC-6 (0.76 μmoles, equivalent to 3 ppm selenium) and compared with animals treated with PBITC or PHITC (0.76 μmoles), respectively (two-way ANOVA, $P < 0.001$); error bars, SE. ISC-4 and ISC-6 reduced tumor development by 50% to 60% compared with PBITC-, PHITC-, or DMSO control-treated mice.

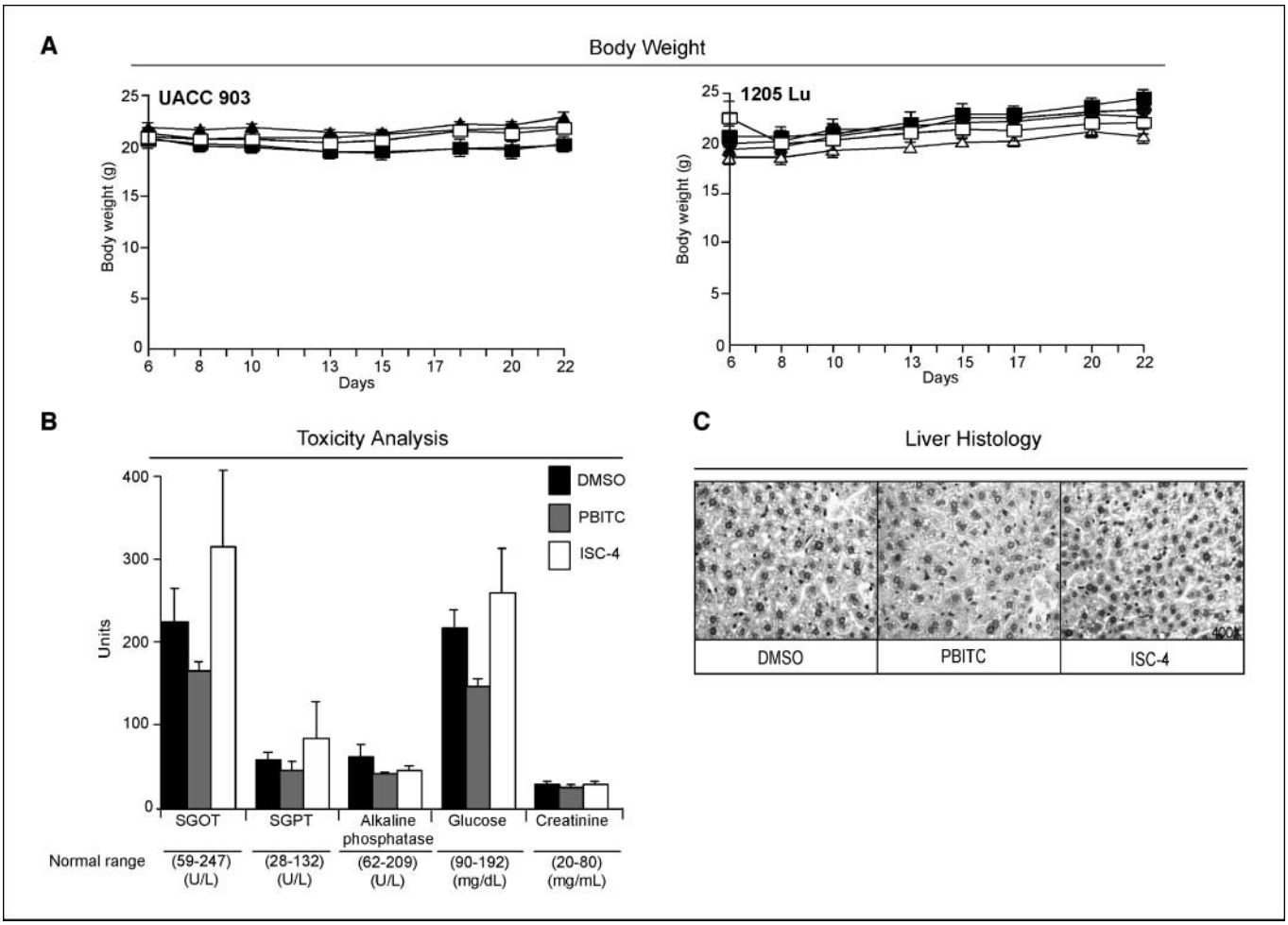


Fig. 4. No major organ related toxicity was associated with isoselenocyanate treatment. *A,* body weight of animals was measured at the time of treatment to ascertain weight related toxicity in animals. No significant changes in body weight were observed, suggesting negligible systemic toxicity. *B,* levels of serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, glucose and creatinine were analyzed in blood collected from animals treated with PBITC, ISC-4, or DMSO vehicle. No significant differences were observed, indicating negligible vital organ – related toxicity; error bars, SE. *C,* histologic analysis of liver tissue shows no liver-associated toxicity following isoselenocyanate treatment. H&E-stained sections (400×) of liver tissue showing no significant differences in liver histology that would be indicative of damage caused following isoselenocyanate treatment.

nude mice. UACC 903 melanoma cells having high Akt3 signaling activity were injected s.c. and six days later when a vascularized tumor had developed, mice were injected i.p. with 2.5 μmoles of each isothiocyanate or 0.76 μmoles of isoselenocyanate (Fig. 3A). While 3-fold less isoselenocyanate was administered, ~50% tumor inhibition was observed at day 24, which indicates enhanced tumor inhibitory effectiveness of selenium-containing analogs. Increasing carbon chain length of isothiocyanates seemed to be less effective at tumor inhibition (Fig. 3A; *left panel*). In contrast, increasing carbon chain length of isoselenocyanates was associated with more effective tumor inhibition ($P < 0.001$, one-way ANOVA; ISC-1 versus ISC-2, ISC-4, ISC-6; Fig. 3A; *right panel*). Thus, PBITC and PHITC reduced tumor development but at concentrations 3-fold higher than corresponding isoselenocyanates. Therefore, 4 to 6 carbon chain isoselenocyanates seemed to be the most robust inhibitors of melanoma tumorigenesis. Based on these findings subsequent studies focused on comparing ISC-4 and ISC-6 with PBITC and PHITC, respectively.

UACC 903 and 1205 Lu melanoma cells having high Akt3 signaling activity were injected s.c. and following six days when tumor angiogenesis had occurred, mice were exposed to 0.76 μmoles representative isothiocyanate compound PBITC versus ISC-4 or PHITC versus ISC-6, three times per week, and tumor development was measured (Fig. 3B and C). Animals were also weighed to ascertain possible toxicity. Whereas PBITC and PHITC are ineffective at reducing the tumor burden of UACC 903 (Fig. 3B) or 1205 Lu (Fig. 3C) at this concentration, ISC-4 and ISC-6 led to significant ($P < 0.001$, two-way ANOVA) reductions in tumor size beginning from day 13 for UACC 903 cells or from day 10 for 1205 Lu cells. Thus, isoselenocyanates are effective at reducing melanoma tumor development by 50% to 60% at significantly lower concentrations than corresponding isothiocyanates, which is similar to siRNA-mediated inhibition of Akt3 (Fig. 1A).

Synthetic isoselenocyanate compounds causes negligible organ related toxicity following systemic administration. Systemic toxicity of PBITC, PHITC, ISC-4, or ISC-6 administration was evaluated in nude mice. Body weights of mice treated with isothiocyanate or isoselenocyanate compounds compared with control DMSO vehicle showed no significant differences between groups (Fig. 4A). Furthermore, blood parameters

(serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, blood urea, glucose, and creatinine) indicative of systemic toxicity did not detect significant liver-, kidney-, or cardiac-related toxicity (Fig. 4B). Levels of cellular metabolites basal urea nitrogen, creatinine, and glucose in animals were also not significantly different between ISC-4- or PBITC-treated and control animals. Histologic examination of H&E-stained vital organ sections, including the liver (Fig. 4C), revealed that ISC-4 treatment did not significantly alter cell morphology or structure of kidney, adrenal, lung, spleen, heart, pancreatic, or intestinal tissue (data not shown). Similar results were observed following treatment with ISC-6 in animals (data not shown). Thus, treatment using synthetic selenium-containing analogs of isothiocyanate ISC-4 or ISC-6 led to negligible associated systemic toxicity with significant therapeutic potential.

Isoselenocyanates decreased Akt3 signaling in cultured melanoma cells and tumors. Cells were next treated with isoselenocyanates ISC-4 and ISC-6, and the effect on Akt3 signaling was examined by Western blotting. Both compounds inhibited Akt3 signaling as shown through decreased pAkt and downstream pPRAS40 levels (Fig. 5A). However, ISC-4 and ISC-6 were effective at lower concentrations, completely inhibiting the pathway at ~10 μmol/L compared with corresponding isothiocyanates requiring ≥15 μmol/L for similar inhibition (Fig. 5A). As reported previously, Akt3 pathway inhibition led to apoptosis, which was indicated by high levels of cleaved poly (ADP-ribose) polymerase (Fig. 5A; ref. 7). Higher cleaved poly (ADP-ribose) polymerase levels were observed at lower concentrations of ISC-4 than corresponding isothiocyanate PBITC, suggesting isoselenocyanate compounds were more effective than corresponding sulfur containing isothiocyanates. Similar Akt pathway inhibition by ISC-4 or ISC-6 (not shown) also occurred for human melanoma cell lines 1205 Lu and WM115 (Fig. 5B).

Western blot analysis of size- and time-matched tumors harvested on day 13 from animals treated with DMSO, PBITC, or ISC-4 also showed significantly decreased phosphorylated (active) Akt ($P < 0.05$, one-way ANOVA) and downstream PRAS40 ($P < 0.001$, one-way ANOVA) in ISC-4 tumor lysates compared with DMSO control or PBITC-treated tumors

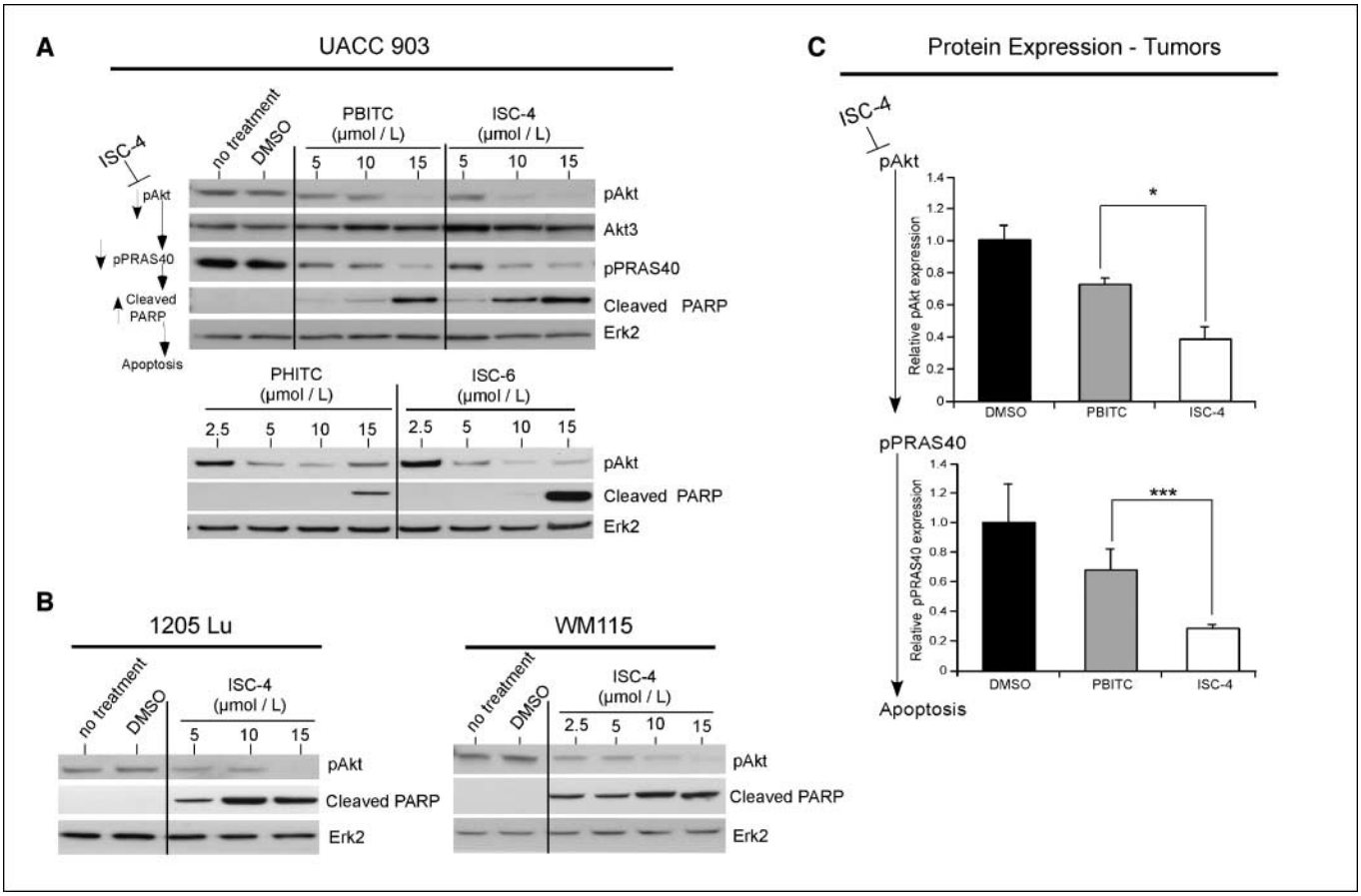
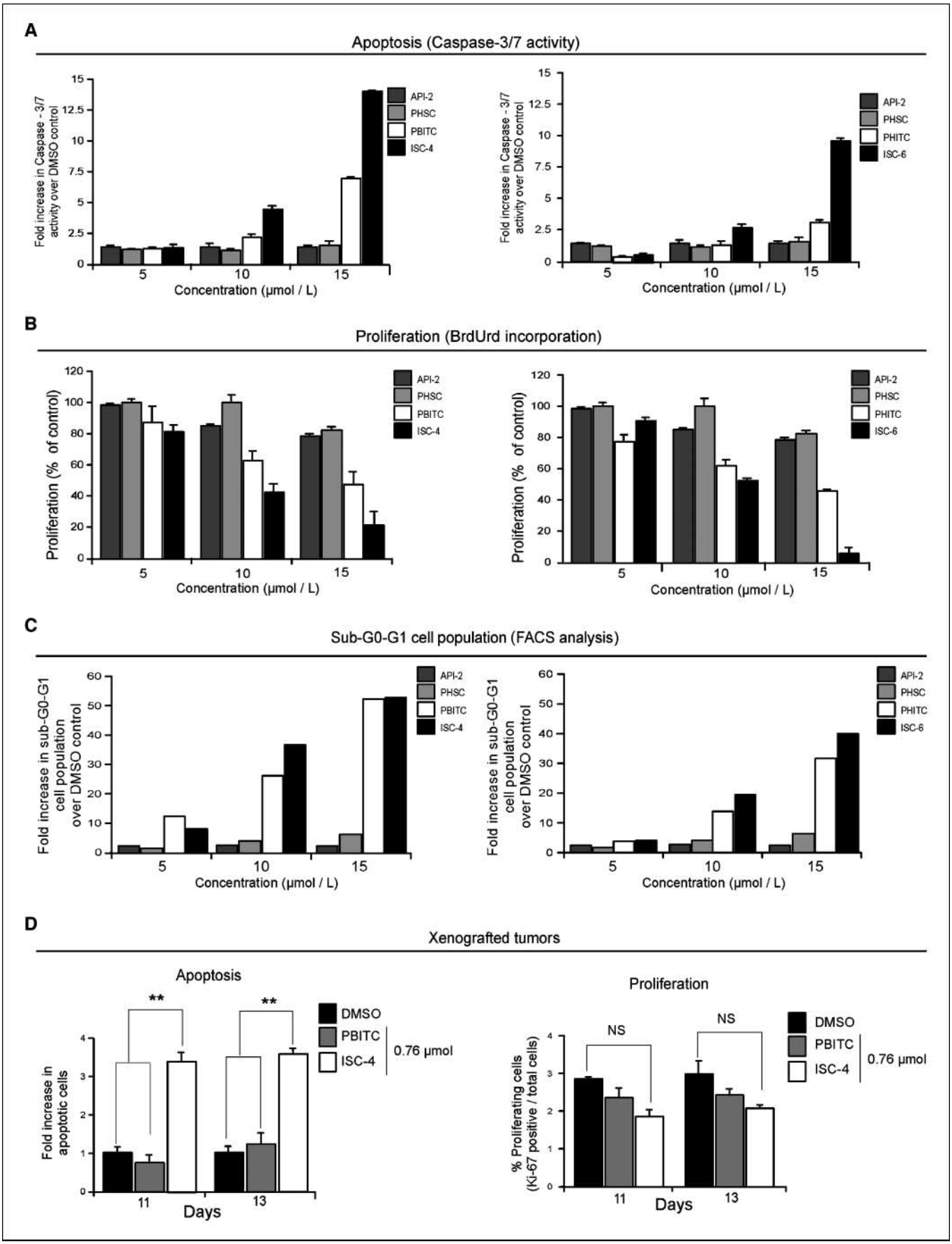


Fig. 5. Inhibition of Akt3 signaling mediated by isoselenocyanates induces apoptosis in melanoma cells. *A*, Western blot analysis of cells treated with ISC-4 or ISC-6 show decreased Akt3 signaling. Melanoma cells (UACC 903,1205 Lu, or WM115) were exposed for 24 h to increasing concentrations (2.5-15 μmol/L) of ISC-4 or ISC-6 and compared with PBITC, PHITC, or DMSO. Western blot analysis measuring activity of the Akt3 signaling pathway shows dose-dependent decrease in pAkt (S473), downstream pPRAS40 (T246), and increase in cleaved poly (ADP-ribose) polymerase, indicating increased cellular apoptosis. Erk2 served as control for equal protein loading. *B*, ISC-4 decreases Akt3 signaling in 1205Lu and WM115 melanoma cell lines. Western blot showing effect of ISC-4 treatment on Akt3 activity in 1205 Lu and WM115 cells. ISC-4 decreases pAkt levels and increases cellular apoptosis levels as indicated by elevated cleaved poly (ADP-ribose) polymerase protein. Erk2 served as a control for equal protein loading. *C*, ISC-4 decreases pAkt and downstream pPRAS40 levels in tumors. Densitometric quantitation of Western blot analysis of tumor protein lysates from animals treated with PBITC or ISC-4 and compared with DMSO vehicle treatment shows decreased relative expression of pAkt and downstream pPRAS40 normalized against α-enolase indicating decreased Akt3 signaling in tumors following treatment (one-way ANOVA, $^{***}P < 0.001$; $^{*}P < 0.05$); error bars, SE.



(Fig. 5C). Thus, isoselenocyanates ISC-4 and ISC-6 were more robust inhibitors of the Akt3 signaling cascade in cultured melanoma cells as well as in xenografted melanoma tumors than corresponding sulfur-containing isothiocyanates.

Isoselenocyanates induced apoptosis in cultured melanoma cells as well as in xenografted melanoma tumors. To identify the underlying mechanism by which isothiocyanates or isoselenocyanates inhibited melanoma cell survival, rates of apoptosis and proliferation were examined following treatment. In contrast to API-2 or PHSC, increasing concentrations of PBITC, ISC-4, PHITC, or ISC-6 led to increased cellular apoptosis (Fig. 6A) and decreased proliferative potential (Fig. 6B) of UACC 903 melanoma cells. ISC-4 or ISC-6 was ~2-fold more effective than PBITC or PHITC at inducing apoptosis and inhibiting cellular proliferation (Fig. 6A and B). Cell cycle analysis of asynchronously growing UACC 903 cells showed a significant increase in the sub-G_0-G_1 population in PBITC- or ISC-4- and PHITC- or ISC-6–treated cells compared with controls (Fig. 6C), which is indicative of cellular apoptosis. Analysis of cells in each stage of the cell cycle, in Supplementary Table, showed a 30% to 40% decrease in the G_0-G_1 phase cells with a 50% to 60% increase in the G_2-M phase cell population. Marginal changes were observed in S-phase cells. The most significant change was an ~15-fold increase in the sub-G_0-G_1 cell population indicating a dramatic increase in cellular apoptosis.

To confirm that a similar mechanism led to tumor inhibition in animals following ISC-4 treatment, rates of apoptosis (TUNEL staining) and proliferation (Ki-67 immunohistochemistry) were compared in size- and time-matched melanoma tumors from ISC-4- or PBITC-treated animals and compared with DMSO vehicle. Tumors harvested on days 11 and 13 from mice treated with ISC-4 showed ~3-fold (Fig. 6D, *right panel*; $P < 0.01$, one-way ANOVA) more TUNEL-positive cells compared with control animals treated with DMSO or PBITC. Slightly fewer proliferating tumor cells were observed in ISC-4–treated tumors compared with PBITC, but this difference was not statistically significant (Fig. 6D, *left panel*; $P > 0.05$, one-way ANOVA). Thus, the superior antimelanoma activity of ISC-4 relative to PBITC seems primarily to be due to an effect on tumor cell apoptosis rather than on cellular proliferation, which is consistent with effects observed following treatment of cultured cells (Fig. 5A and Supplementary Table).

Discussion

For several decades, no substantial progress has been made in developing drugs effective for the long-term survival of patient's with advanced-stage melanoma (33). Current systemic therapies for metastatic disease still achieve only a modest ~20% overall response rates, and the duration of efficacy is typically months and not years (1, 33). The median progression-free survival following initiation of systemic therapy for stage IV melanoma is typically about 1.7 months, and the median survival is 6.2 months (34). Clearly, a pipeline of novel, more effective therapeutics is needed to increase the long-term survival of metastatic melanoma patients, which is the goal of this report.

Targeted agents that inhibit the activity of aberrant melanoma-causing genes have the potential to significantly increase patient survival (1, 9, 11). Agents of this type have been shown to be effective, for example: imatinib targeting receptor-type KIT tyrosine kinase and BCR-ABL tyrosine kinase in chronic myelogenous leukemia and gastrointestinal stromal tumors; bevacizumab targeting vascular endothelial growth factor (VEGF) in colorectal cancer; sunitinib targeting VEGF receptors, FMS-like tyrosine kinase 3, c-KIT, and platelet-derived growth factor in renal cell carcinoma; and sorafenib targeting Raf kinases in renal cell as well as hepatocellular carcinomas (35–39). Thus, it is reasonable to assume that agents could be developed to inhibit proteins deregulated during melanoma development that would be more effective than currently available drugs for treating this disease.

The Akt3 pathway is an important pathway deregulated in ~70% of melanomas, important to therapeutically target alone or in combination with other targeted agents (6–8). A second key pathway is that of the mitogen-activated protein kinase signaling cascade, which is constitutively activated through Ras mutations in 10% to 15% and B-Raf mutations in ~60% of melanomas (40, 41). A T-to-A mutation at nucleotide 1799 of B-Raf leads to substitution of a valine for a glutamic acid at codon 600 (V600E) in exon 15 in the vast majority of melanomas in which B-Raf is mutated (41). This alteration is acquired during development of sporadic melanomas and not inherited (41). Because B-Raf is the most mutated gene in melanomas, it is an attractive therapeutic target (40). Sorafenib, which was identified as a Raf kinase inhibitor, was initially hoped to be effective for treating melanoma (10, 40). However, off-target effects have limited its efficacy for treating melanoma. Sorafenib inhibits Raf, but it also decreases activity of VEGFR1, VEGFR2, VEGFR3, platelet-derived growth factor receptor α, Flt-3, p38, c-Kit, and fibroblast growth factor receptor 1 (42). Therefore, although it targets constitutively active mutant ^{V600E}B-Raf present in ~60% of metastatic melanomas, it is ineffective because its primary mechanism of action is as an angiogenesis inhibitor and not as a regulator of cellular

Fig. 6. Isoselenocyanates increase cellular apoptosis in cultured cells and melanoma tumors. *A,* isoselenocyanates induce apoptosis in cultured melanoma cells. Levels of caspase-3/7 activity in cultured melanoma cells exposed to ISC-4 or ISC-6 were compared with PBITC, PHITC, API-2, and PHSC using the Apo-ONE homogeneous caspase-3/7 assay kit. Results show significant dose-dependent increases in caspase-3/7 activity relative to DMSO vehicle–treated cells. Results represent the average of three independent experiments. *B,* isoselenocyanates reduces proliferation of cultured melanoma cells. Proliferating UACC 903 cells, using a BrdUrd ELISA kit, were measured following 24-h treatment with 5-15 μmol/L ISC-4 or ISC-6 and compared with PBITC, PHITC, API-2, and PHSC. Results show a dose-dependent decrease in proliferating cells with maximal inhibition occurring following isoselenocyanate treatment. Values represent the average of the percentage of control DMSO–treated cells from three independent experiments. *C,* isoselenocyanates increase sub-G_0-G_1 cell population indicating increased cellular apoptosis. Asynchronously growing UACC 903 cells were treated with ISC-4, ISC-6, PBITC, PHITC, API-2, or PHSC, and 24 h later, cells stained with propidium iodide were analyzed for cell cycle distribution using a FACScan analyzer. ISC-4 or ISC-6 treatment significantly increased the sub-G_0-G_1 cell population indicating apoptotic cells. Results represent the average of two independent experiments. *D,* isoselenocyanate treatment increases levels of cellular apoptosis in melanoma tumors. Rates of apoptosis and proliferation in size- and time-matched tumors from mice treated i. p. with ISC-4 (3 ppm equivalent to 0.76 μmoles), starting 6 d after s.c. injection of cells and on alternate days thereafter up to day 13, were compared with mice treated with PBITC (0.76 μmoles) or DMSO (50 μL). Results show a 3-fold increase in number of apoptotic cells following treatment of UACC 903 tumors with ISC-4 on days 11 and 13 compared with PBITC or DMSO control. No statistically significant difference was observed in proliferation rate. Values represent means from two separate experiments with four to six fields analyzed from each of six tumors per experiment. (one-way ANOVA, ** $P < 0.01$; NS, non significant); error bars, SE.

proliferation, which is required for effective melanoma inhibition when targeting this aberrant signaling cascade (9, 10, 43). More specific B-Raf inhibitors are being developed and evaluated to circumvent these limitations, but clinical efficacy is currently unknown (11).

Inhibitors of the Akt3 pathway were developed in this report by initially screening a natural product library for candidate antineoplastic compounds that inhibited this signaling cascade. The screen was based on reports showing that targeting Akt3 signaling significantly reduced the tumorigenic potential of melanoma cells (6, 7, 15). Naturally occurring isothiocyanates were identified as potential inhibitors of Akt3 signaling. Numerous naturally occurring compounds exhibiting antineoplastic properties are being exploited as potential chemotherapeutic agents (44, 45). Some are well-established components of standard systemic chemotherapeutic regimens, such as the taxanes, vinca alkaloids, and camptothecins (44, 46, 47). However, no successful agent or combination of agents has been identified that dramatically extends melanoma patient survival (33). Thus, naturally occurring agents targeting key signaling pathways, such as Akt3, could be important breakthroughs for more effective melanoma therapies.

Whereas naturally occurring isothiocyanates were found to inhibit Akt3 signaling, impractical quantities were required for melanoma antitumor activity. Therefore, replacing the sulfur group with selenium and lengthening the carbon chains was evaluated to enhance potency as therapeutic agents. The resulting family of compounds, called isoselenocyanates, had greater efficacy killing cultured cells as well as inhibiting tumor development in animals compared with sulfur-containing isothiocyanates. Two isoselenocyanates, ISC-4 and ISC-6, had particularly robust antimelanoma activity with enhanced potency due primarily to enhanced tumor cell apoptosis following treatment. Thus, isoselenocyantes represents a significant development in the natural product drug pipeline by targeting a key signaling cascade deregulated in ~70% of melanomas.

Incorporating selenium into the structure of isoselenocyanates is a further significant development. Selenium plays a role in cancer chemoprevention, but the exact mechanistic basis for inhibition remains to be identified (28, 29, 48, 49). Several clinical trials are examining the role of selenium in the prevention of colorectal cancer (NCT00078897), breast cancer (NCT00555386), lung cancer (NCT00008385), and bladder cancer (NCT00553345). Furthermore, incorporating selenium into the structure of drugs, as in the case of isoselenocyanates, can increase compound potency making ineffective agents better therapeutics. Recently, a selenium-containing analog of the inducible nitric oxide synthase inhibitor PBIT, called PBISe, which is ineffective at killing melanoma cells, was made >10-fold more potent by incorporating selenium into its structure to more effectively decrease melanoma tumor development in animals (50). Thus, incorporating selenium into the structure of cancer therapeutics is one feasible approach to increase the tumor inhibitory efficacy of therapeutic agents.

Because control compounds containing selenium had little effect on melanoma cell survival, isoselenocyanate-mediated inhibition of melanoma is independent of selenium, suggesting that the structure of the compound in combination with selenium is necessary for enhanced inhibitory activity. Compared with the chemopreventive effects of natural selenium-enriched products, isoselenocyanates are unique in that the selenium-containing compounds target Akt3 signaling in melanoma to promote apoptosis, block the growth of tumors, and are associated with negligible toxicity at biologically effective doses. Therefore, isoselenocyanates represent a promising adjunct to rational, targeted, single-agent or perhaps multiagent therapy for advanced melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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