Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 1/1/2009 – 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** John Anthony, MPA

4. **Grant Contact Person's Telephone Number:** 814-935-1081

5. **Grant SAP Number:** # 4100047645

6. **Project Number and Title of Research Project:** 18 - Moving Experimental Cancer Therapeutics from the Research Bench to the Clinic

7. **Start and End Date of Research Project:** 5/1/2009-12/31/2012

8. **Name of Principal Investigator for the Research Project:** Gavin P. Robertson, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 1,435,515

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Robertson, G	Principal Investigator	22% Yr 1; 2% Yr 2; 2% Yr 3	55,292.85
Sharma, A	Asst. Professor	9% Yr 1; 35.3% Yr 3	60,909.02
Inamdar, G	Post-Doctoral Fellow	21% Yr 1	8,617.96
Chandagaludore, R	Post-Doctoral Fellow	49% Yr 3	20,726.52
Klinger, M	Research Technician	8% Yr 3	4,413.50

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Robertson, G	Principal Investigator	2%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
FACS III	One of most advanced cell sorters on the Market	$607,685
Two -86C Upright Freezers	Storage	$18,204
Zetasizer Namo ZS System	Size and charge of nanoparticles for synergy experiments	$53,255
HPLC System	Compound measurements in biological samples	$29,598
Real time PCR Detection System	Measuring RNA levels in realtime	$48,717
Highlight FAP 30:80-820nm Laser (Dell Optiplex 3010 PC w/monitor for Laser	Laser treatment of cells for nanoparticle synergy experimentation	$21,750
Chemidoc XRS Imaging System	Imaging of fluorescence, colorimetry, densitometry, chemiluminescence, and chemifluorescence cells.	$28,156
Konica Automatic Film Processor	Processing blots for western blotting	$22,800
Oriel Solar Simulator	Treating samples with UV light	$37,598
BX 61 Microscope system	Imaging of Florescent and Non-Florescent cells	$78,204
Centrifuge	Spinning out cells for culture	$8,941
Rotavapor	Compound synthesis	$9,959

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No_____X____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X___ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Establishing Bioactive Serum and Toxicity Levels for Nanolipolee-007	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	12/2011	$232,566.80	$232,566.80

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

R01 or other R- type applications.

12. Future of Research Project. What are the future plans for this research project?

Test drugs in patients

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				2
Female				
Unknown				
Total				**2**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				2
Unknown				
Total				**2**

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				2
Unknown				
Total				**2**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This project has enabled a better translational research potential, and the equipment purchased will benefit not only this project but all of the Departments and researchers in the Cancer Institute.

The Becton Dickinson FACSAria III SORP is one of the most advanced cell sorters currently on the market, capable of high speed separation using a total of 17 unique parameters (15 fluorescent wavelengths plus cell size and granularity) for collection of live cells. The presence of 4 high-powered lasers to excite the different fluorescent labels in combination with powerful software promotes excellent separation of the signals, such that small populations of unique cell types amidst large populations of mixed cell types can be specifically identified, captured, and studied. In addition, this instrument allows simultaneous collection of up to 4 different live cell populations. This particular model includes a number of safety features that contain the aerosol created by high-speed sorting and is enclosed in a biosafety cabinet, allowing the safe separation of cells from bio-hazardous samples (up to BSL-2+ designation, which was never previously possible at the Penn State College of Medicine).

Replacement of the old MoFlo cell sorter with the new FACSAria SORP instrument has greatly increased the sorting capabilities within the Flow Cytometry Core Facility as evidenced by increased use of the instrument by the research community. Over 15 different investigators have already used the instrument since it came on-line in October of 2012 and the instrument is now routinely booked almost every day for two weeks in advance. Value added includes the ability to perform experiments using BL-2+ samples, increased options for cell markers used to identify subpopulations of cells and decreased costs to the investigator due to the ability to simultaneously collect up to 4 different populations from the same starting sample which saves both material and manpower. One example of current applications includes sorting of tumor stem cells into unique populations in order to identify the earliest precursors that promote breast cancer progression. A second important study uses this instrument to collect stem cells that have been genetically modified to develop into immune cells capable of either rejecting tumors in mice, or preventing autoimmune disease progression. Yet another study is isolating populations of genetically modified tumor cells in order to identify the role of tumor suppressor genes in the development of childhood leukemia. These latter two studies were only made possible by the high level of aerosol control that increases the safety of these types of experiments. Thus, the FACSAria SORP has made an immediate major impact on the research community at Penn State Hershey by providing new and enhanced cell collection capabilities for the purpose of identifying mechanisms of disease progression and new approaches to treatment.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____X_____ No_____

If yes, please describe the collaborations:

Melanovus Oncology – Commercialization partner

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16

months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

AIMS:

Clinicians treating cancer believe that agents targeting key pathways deregulated in cancer cells are necessary for more effectively treating these diseases. Unfortunately, relatively few agents are available in the clinic to treat cancer in this manner. Malignant melanoma is a prime example of a devastating disease needing more effective agents for treatment of metastatic disease. The hope is that targeted agents inhibiting the activity of aberrantly behaving proteins would be more effective than those untargeted drugs currently available to patients. To develop better cancer therapies to treat melanoma, The Penn State Melanoma Therapeutics Program is identifying genes causing the disease, developing drugs to target these genes and drug delivery systems to get the agents more effectively to the cancer cells, and finally testing the agents in clinical trials in patients. However, the biggest hurdle is the final step, which involves testing the agents in the clinic. Therefore, the objective of this project is to develop the organizational structure necessary to move agents developed within the Melanoma Therapeutics Program from the preclinical to clinical arena.

The Penn State Melanoma Therapeutics Program has a Therapeutic Drug Portfolio of ~20 experimental therapeutic compounds at various stages of development. Unfortunately, agents proceed through preclinical development at which point progress halts. The purpose of this project is to establish the necessary expertise and infrastructure to more rapidly move these compounds to the clinic. This involves developing interactions with companies to make clinical grade compound for testing in patients; undertaking necessary toxicological and pharmacological evaluation for an Investigational New Drug (IND) application; and interacting with the Food and Drug Administration (FDA) to enable experimental agents to obtain IND status so that they can be evaluated in the Pennsylvania State University (PSU) Cancer Institute Clinics in phase I trials. Accomplishing these objectives for one of the compounds from the current melanoma drug portfolio is the objective of this project.

Clinicians treating cancer believe that agents targeting key pathways deregulated in cancer cells are necessary for more effectively treating these diseases. Unfortunately, relatively few agents are available in the clinic to treat cancer in this manner. Malignant melanoma is a prime example of a devastating disease needing more effective agents for treatment of metastatic disease. The hope is that targeted agents inhibiting the activity of aberrantly behaving proteins would be more effective than those untargeted drugs currently available to patients. To develop better cancer therapies to treat melanoma, The Penn State Melanoma Therapeutics Program is identifying genes causing the disease, developing drugs to target

these genes and drug delivery systems to get the agents more effectively to the cancer cells, and finally testing the agents in clinical trials in patients. However, the biggest hurdle is the final step, which involves testing the agents in the clinic. Therefore, the objective of this project is to develop the organizational structure necessary to move agents developed within the Melanoma Therapeutics Program from the preclinical to clinical arena. Development of an agent known as ISC-4, which inhibits the Akt3 kinase deregulated in ~70% of melanomas will be emphasized. Therefore, the *objective of this project* is to undertake the necessary characterization to move ISC-4 from the preclinical arena to the clinic. First, a large batch of GMP grade ISC-4 is required, which will be compared to currently used smaller laboratory generated batches. By-products and physical characteristic will be determined. Next, efficacy for inhibiting tumor development using the larger synthesis scale will be compared to smaller scale synthesis together with toxicological evaluation. Second, toxicology as well as pharmacokinetic and pharmacodynamic properties of ISC-4 in a second animal model will be evaluated. Collectively, these studies will form the foundation for an IND from the FDA for ISC-4. Once these data are obtained, The Penn State Cancer Institute plans to test the compound's toxicity in humans as well as determine any efficacy in a phase I melanoma clinical trial. Note: A phase I trial testing of the agent in melanoma is not the objective of this project, rather it is to successfully obtain IND status for the compound. Thus, the *central hypothesis* for these studies is that targeting Akt3 signaling using ISC-4 would be an effective targeted approach for inhibiting melanoma development. This discovery would be highly significant, evaluating the therapeutic implications of targeting a major signaling pathway promoting melanoma development.

One person in the United States dies from melanoma every hour. Currently, no effective treatment exists for patients suffering from the metastatic stages of this disease. In spite of the widely appreciated magnitude of the problem, there is still a critical gap in knowledge regarding key deregulated signaling pathways causing melanoma and therapies specifically targeted to correct these defects to inhibit tumorigenesis and metastasis. *Availability of ISC-4 as a melanoma therapeutic targeting Akt3 signaling in melanoma has the potential of leading to a more effective rationally targeted therapeutic agent for melanoma patients.* Therefore, as a direct outcome of the project, it is expected that a scaled-up clinical batch of ISC-4 will be generated; the compounds physiochemical and biological characteristics will be evaluated in preclinical models and compared to small-scale lab generated batches. Second, toxicity as well as pharmacokinetics and pharmacodynamics will be determined in a second animal model, which is critical data necessary to apply for an IND for ISC-4 from the FDA so that the compound can be tested in phase I clinical trials. Clinical availability of ISC-4 is predicted to have a significant positive impact on the currently poor prognosis faced by advanced-stage melanoma patients by contributing to availability of more effective therapies, which would increase the length and quality of life for melanoma patients. Therefore, the positive impact for cancer patients suffering from melanoma will be significant.

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First, a 1 kg of ISC-4 has been synthesized and compared to smaller laboratory generated batches. HPLC, NMR and Mass Spec have been used to verify the identity and purity of the ISC-4. Measuring of the efficacy of the ISC-4 drug to kill cultured cancer cells following synthesis at a larger scale, has been compared to this agent synthesized in smaller batches and shown to be equally effective, resulting in similar IC_{50} values. Second, efficacy for inhibiting cutaneous tumor development using the larger synthesis scale together with toxicological evaluation has been evaluated. The chemopreventive efficacy of topical ISC-4 was evaluated in a laboratory-generated human skin melanoma model containing early melanocytic lesions or advanced-stage melanoma cell lines and in animals containing invasive xenografted human melanoma. Cumulative topical application of ISC-4, reduced melanocytic or melanoma developed in the skin model by 80-90% and decreased tumor development in animals by ~80%. Histological examination of ISC-4 treated skin showed no obvious damage to skin cells or skin morphology and treated animals did not exhibit markers indicative of major organ related toxicity. Mechanistically, ISC-4 prevented melanoma by decreasing Akt3 signaling leading to a 3-fold increase in apoptosis rates. Thus, topical ISC-4 can prevent melanoma in preclinical models and has potential to profoundly impact melanoma incidence and mortality rates in humans if similar chemopreventive results are observed. With this proof of principal demonstration, the goal is now to develop a topical formulation, which would be evaluated in clinical trials in humans to prevent melanoma.

Detailed description

The results of this experimentation are detailed in the sections that follow under headings detailing the discoveries.

Targeting Akt3 signaling in malignant melanoma using isoselenocyanates

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.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).

Figure 1

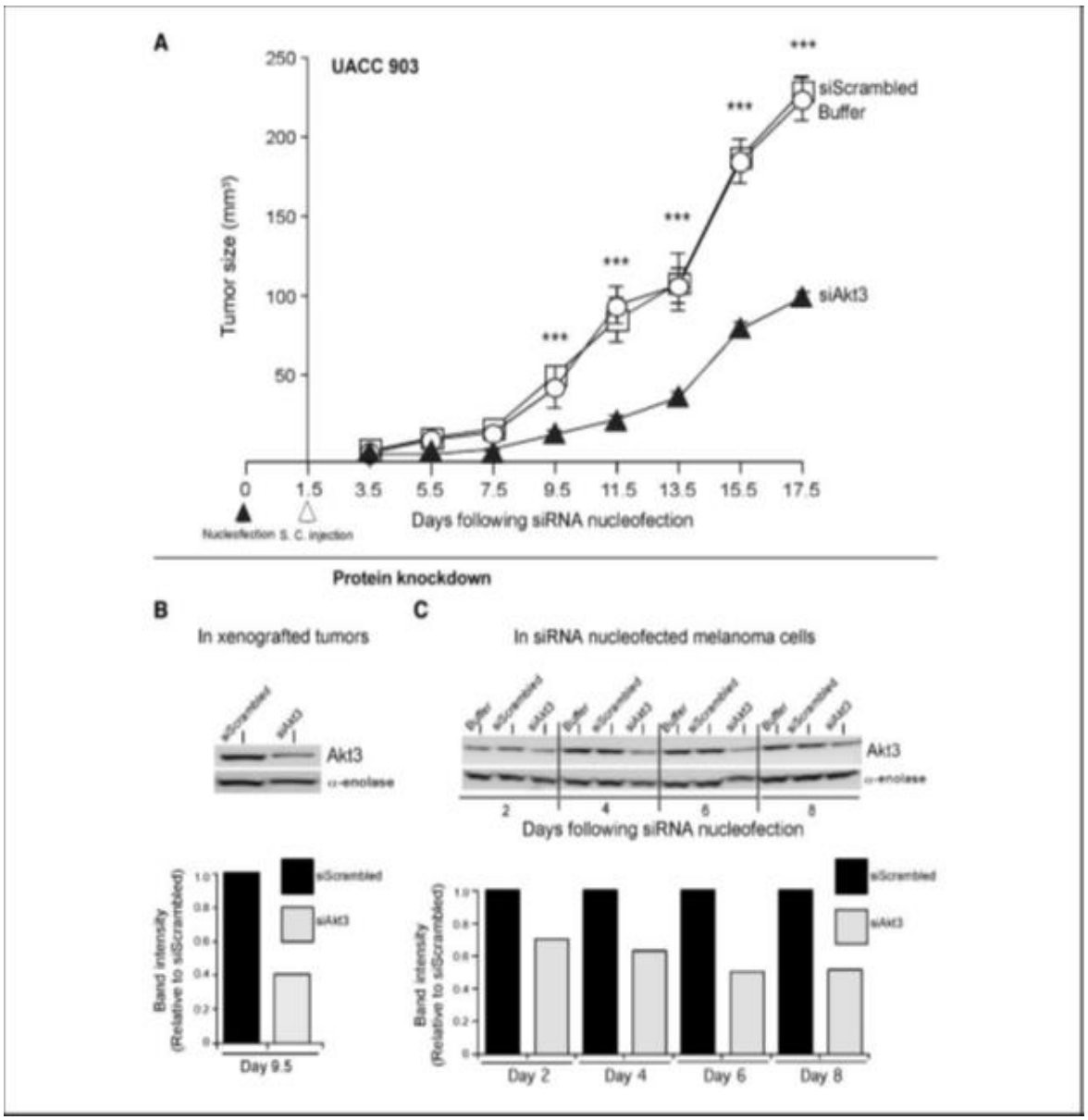


Fig. 1. siRNA-mediated inhibition of Akt3 expression/activity inhibits melanoma tumor development. A, siRNA (100 p moles) against Akt3 (E), scrambled siRNA (5), or nucleofection buffer (o) were introduced into UACC 903 (1106) melanoma cells and 36h later viable cells were injected s.c. into nude mice. 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. a-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. a-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 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 IC50 of the respective compounds. Figure 2B shows are presentative examples 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-Dribofuranosyl-1,4,5,6,8-penta azaacenaphthylen-3-amine; 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 IC50 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 IC50 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.

Figure 2

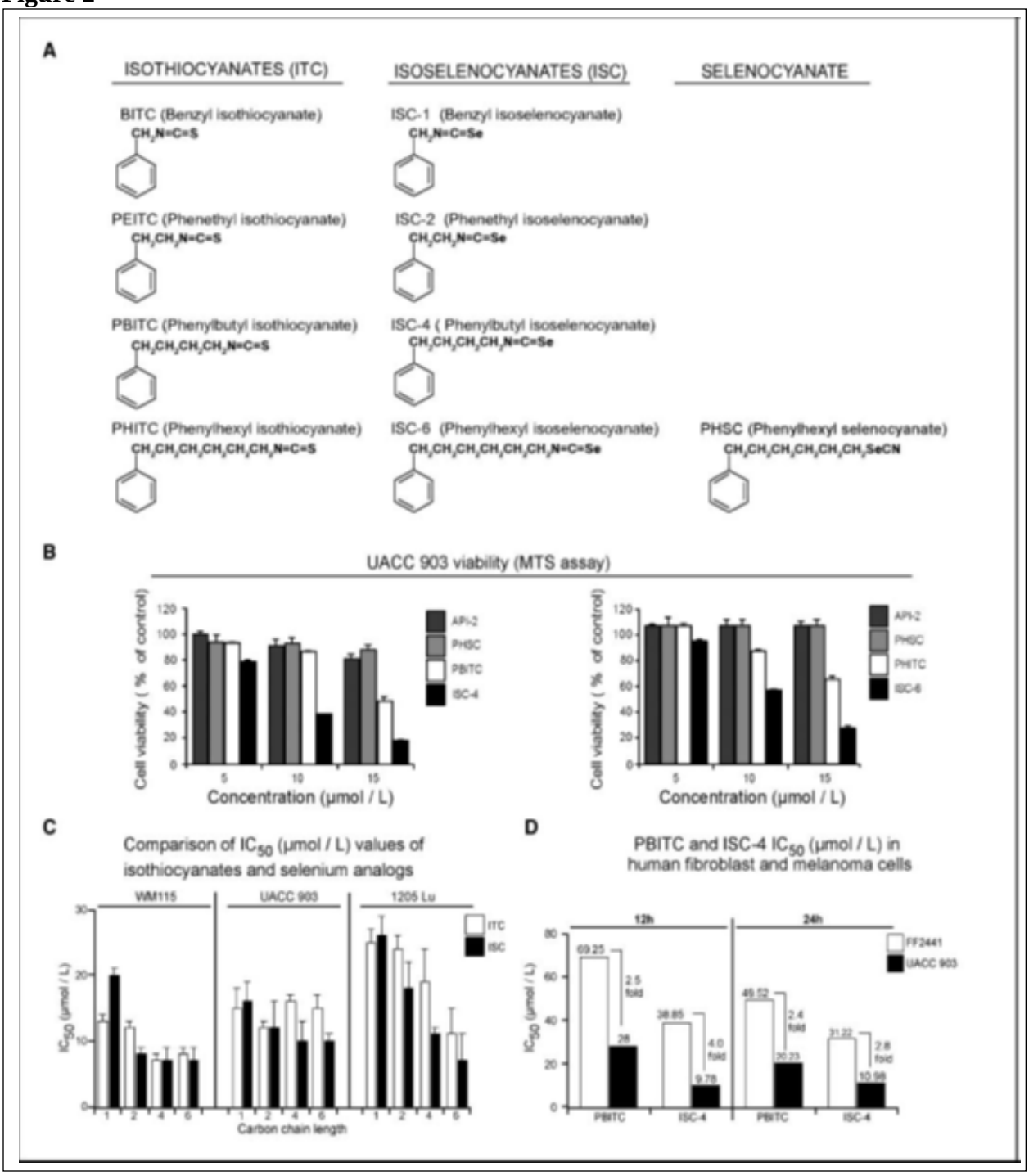


Fig. 2. Characterization of isothiocyanates and isoselenocyanates as inhibitors of melanoma. A, structures of isothiocyanates and selenium-containing isoselenocyanates. Chemical structures of isothiocyanates containing 1(benzyl), 2 (phenethyl), 4 (phenylbutyl), and 6(phenylhexyl) carbon spacers and corresponding 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 IC50 (µmol/L) values plotted against carbon chain length. 5 103 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. 5 103 normal human fibroblasts (FF2441) or metastatic melanoma cells (UACC 903) were plated in 96-well plates in 100 L DMEM media containing 10% FBS and grown for 72 and 36h, respectively. Exponentially growing cells were treated with increasing concentrations (2-100 µmol/L) of ISC-4 or PBITC for 12 and 24 h, and IC50 (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.

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 IC50 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 nude mice. UACC 903 melanoma cells having high Akt3signaling activity were injected s.c. and six days later when a vascularized tumor had developed, mice were injected i.p. with 2.5 Amoles of each isothiocyanate or 0.76 moles of isoselenocyanate (Fig.3A). While 3-fold less isoselenocyanates 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 to0.76 moles 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).

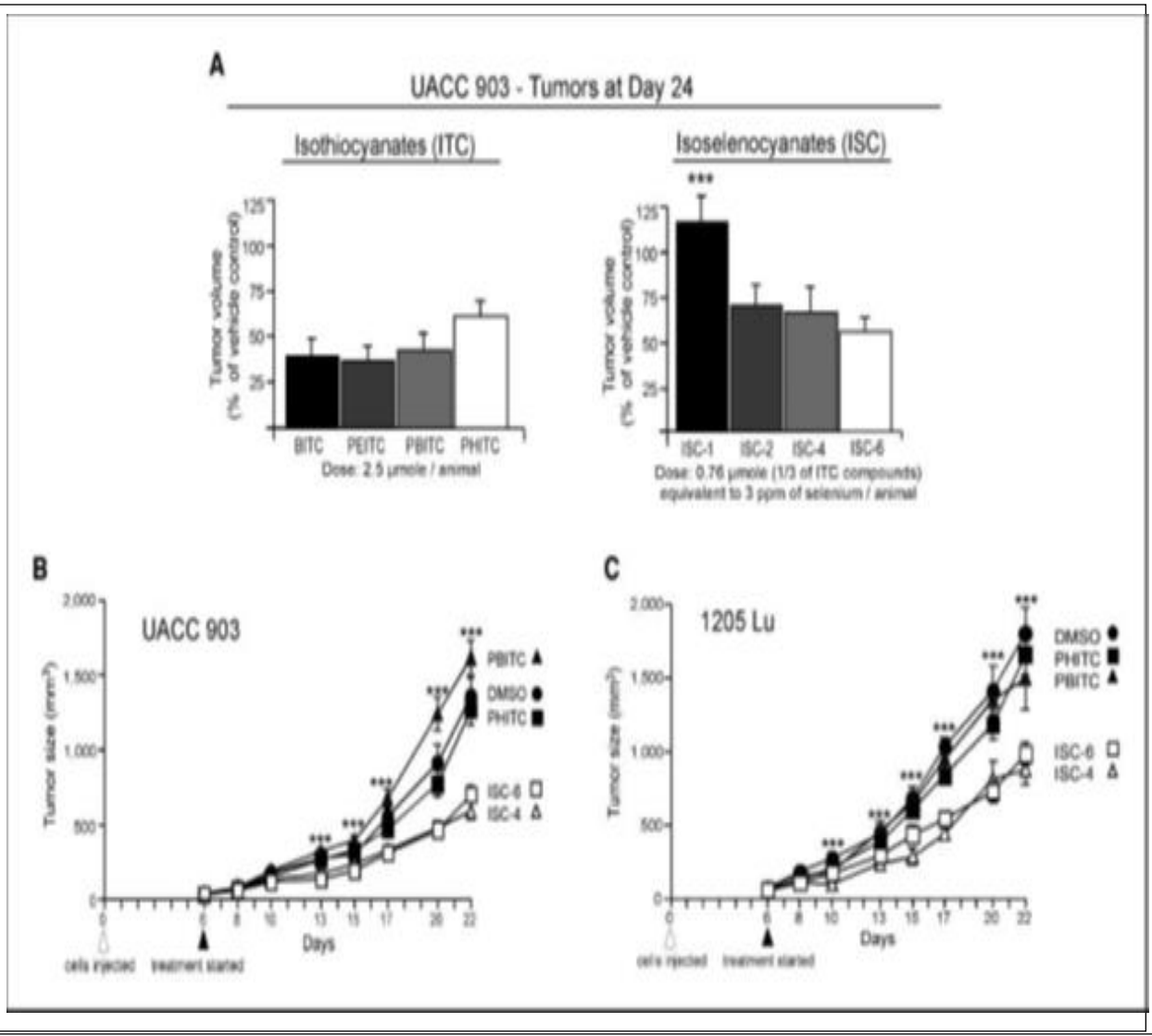


Fig. 3. Isoselenocyanates are effective inhibitors of melanoma tumor development. A, Isoselenocyanates inhibit melanoma development at concentrations 3-fold less than corresponding isothiocyanates. Effect of isothiocyanates and isoselenocyanates on melanoma tumor development was measured by s.c. injection of 5 million UACC 903 cells and after 6d when small vascularized palpable tumors were observed, mice were treated i. p. with isothiocyanates (2.5 moles) or isoselenocyanates (0.76 moles, 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 isoselenocynate than isothiocyanate 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 6d , mice were treated i. p. 3 times per week with ISC-4 or ISC-6 (0.76 moles, equivalent to 3 ppm selenium) and compared with animals treated with PBITC or PHITC (0.76 moles), 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.

Synthetic isoselenocyanate compounds causes negligible organ related toxicity
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. 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.

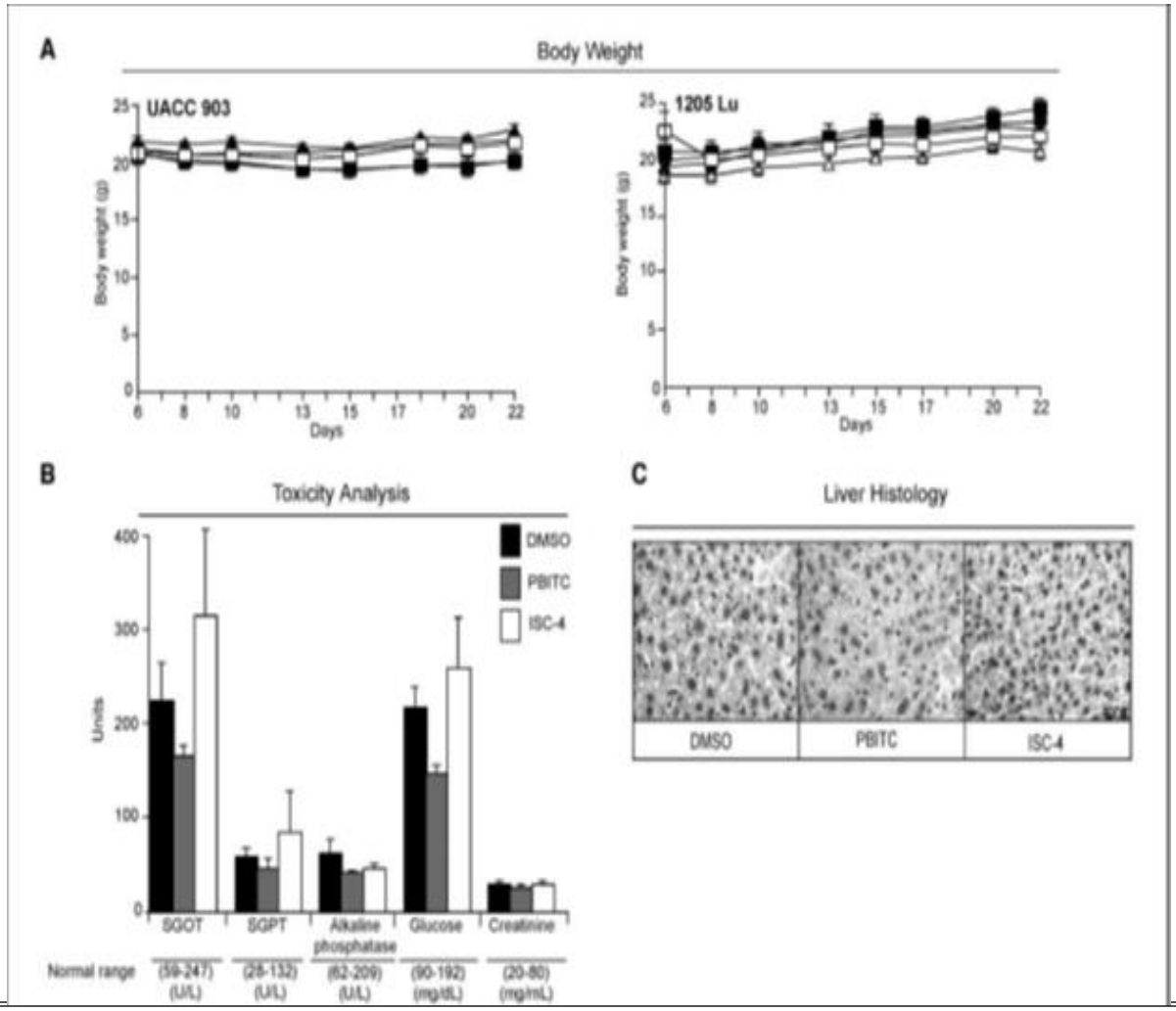


Fig. 4. No major organ related toxicity was associated with isoselenocyanate treatment. A, body weight of animals was measured at the time of treatment to ascertain weight related toxicity in animals. No significant changes in body weight were observed, suggesting negligible systemic toxicity. B, levels 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 (400X) of liver tissue showing no significant differences in liver histology that would be indicative of damage caused following isoselenocyanate treatment.

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 Akt3signaling 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 f10 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 downstreamPRAS40 ($P < 0.001$, one-way ANOVA) in ISC-4 tumor lysates compared with DMSO control or PBITC-treated tumors(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-G0-G1 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 showed a 30% to 40% decrease in the G0-G1 phase cells with a 50% to 60% increase in the G2-M phase cell population. Marginal changes were observed in S-phase cells. The most significant change was an 15-fold increase in the sub-G0-G1cell 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 anti melanoma 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).

Figure 5

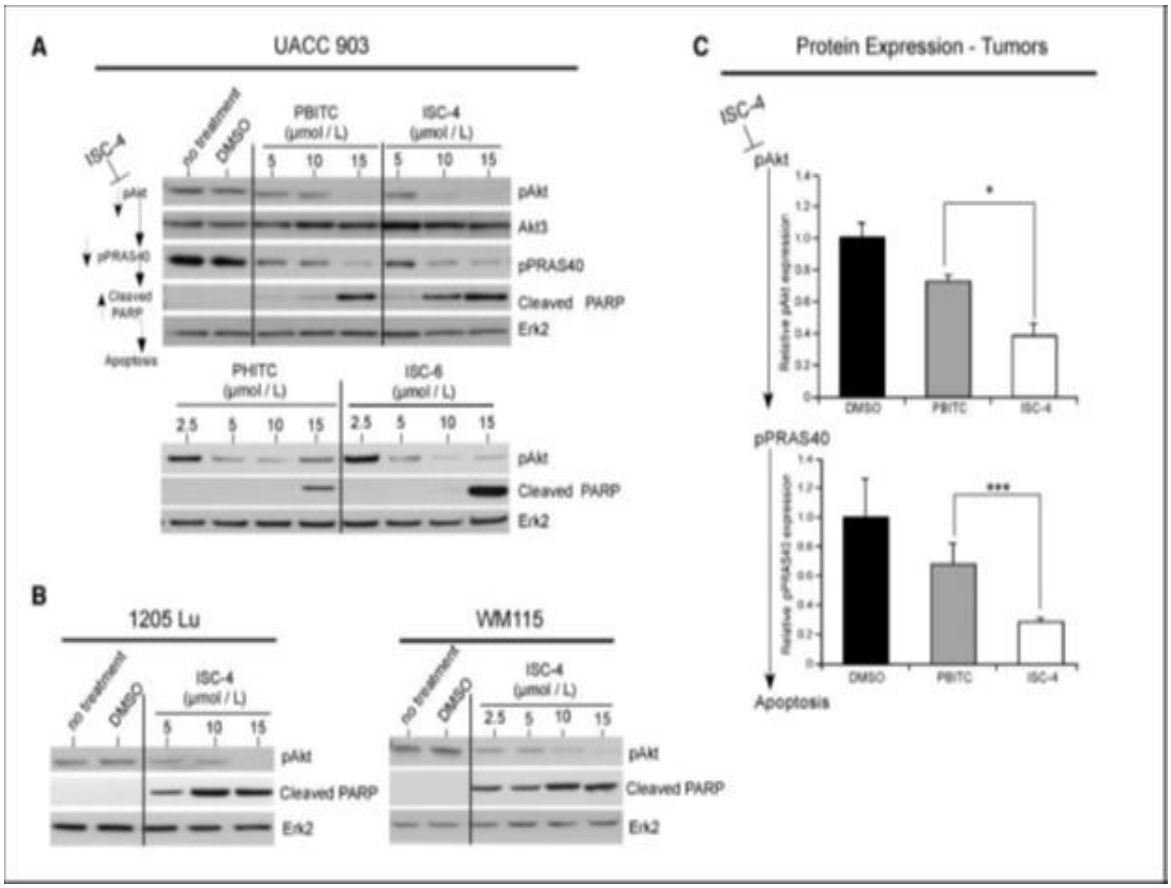


Fig. 5. Inhibition of Akt3 signaling mediated by isoselenocyanates induces apoptosis in melanoma cells. A, Western blot analysis of cells treated with ISC-4 or ISC-6 show decreased Akt3 signaling. Melanoma cells (UACC 903,1205 Lu, orWM115) were exposed for 24 h to increasing concentrations (2.5-15 µmol/L) of ISC-4 or ISC-6 and compared with PBITC, PHITC, or DMSO. Western blot analysis measuring activity of theAkt3 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 in1205Lu and WM115 melanoma cell lines. Western blot showing effect of ISC-4 treatment on Akt3 activity in1205 Lu andWM115 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 a-enolase indicating decreased Akt3 signaling in tumors following treatment (one-way

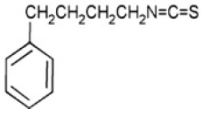
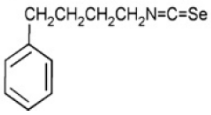
Melanoma Chemoprevention in Skin Reconstructs and Mouse Xenografts Using Isoselenocyanate-4.

Other than sunscreen, which acts as a physical barrier to prevent UV rays from reaching skin cells and thereby preventing some skin cancers, no agent is available to stop melanoma by targeting the genes whose deregulation causes the early stages of this disease. Dacarbazine, which is nonspecific DNA alkylating agent, is an approved chemotherapeutics for advanced stage melanoma but is relatively ineffective at treating melanoma. This is equally true of other available therapeutic strategies for metastatic melanoma including immuno-, radio-, and chemotherapy. Although surgical excision can eliminate melanomas if lesions are removed at the earliest stages of the disease, a significant numbers are not being detected or excised early, leading to rising incidence and mortality rates. Therefore, novel agents are needed to augment existing preventive strategies and one approach is the development of topical agents targeting key pathways regulating early melanocytic lesion development. The Akt3 signaling pathway plays a central and specific role in early melanocytic lesion development by decreasing inhibitory mitogen activated protein (MAP) kinase pathway activity and also by deregulating apoptosis. Although 3 mammalian isoforms of Akt, Akt1/PKBa, Akt2/PKBb, and Akt3/PKBg, have been identified, Akt3 s the predominantly active isoform in early melanocytic lesions and advanced melanomas. It has a very low mutation rate and is preferentially activated in approximately 70% of melanoma patients because of increases in gene copy number and/or loss of a negative regulatory phosphatase called PTEN. Increased Akt3 activity promotes melanocytic lesion development by decreasing both V600EB-Raf activity in the MAP kinase pathway and the apoptotic sensitivity of melanoma cells, mediated throughcaspase-3. Thus, the Akt3 signaling pathway is deregulated in the majority of melanomas, making it a promising therapeutic target, which, if inhibited, could correct the apoptotic defect in melanocytic lesions, thereby preventing this disease. Isothiocyanates were identified as inhibitors of Akt3signaling in melanoma cells from a screen of natural products, which inhibited this pathway. Isothiocyanates are naturally occurring compounds found in cruciferous vegetables having anticancer properties, providing protection against murine tumorigenesis induced by environmental carcinogens such as polycyclicaromatic hydrocarbons and nitrosamines. Certain studies suggest isothiocyanates can act by inhibiting the phosphoinositide 3-kinase pathway. Unfortunately, isothiocyanates had low chemotherapeutic potency on melanoma cells requiring high concentrations for therapeutic efficacy, which made these compounds unsuitable therapeutic or preventive agents. To overcome this limitation, more potent analogues such as isoselenocyanates were developed using the isothiocyanate backbone but increasing the alkyl chain length and replacing sulfur with selenium. Selenium was incorporated into the structure, as it can be an effective chemopreventive agent and its deficiency occurs frequently in cancer patients including those with a diagnosis of metastatic melanoma. Isoselenocyanates had improved therapeutic efficacy for killing cultured melanoma cells or inhibiting tumor development in animals when administered systemically. However, efficacy for preventing cutaneous melanocytic lesion development or for topical applications has not been evaluated. In this study, the chemopreventive effect of isoselenocyanate-4 (ISC-4) on melanocytic lesion development in skin has been evaluated. Topically applied ISC-4 inhibited melanocytic lesion development in laboratory-generated and mouse skin by decreasing Akt3 signaling to trigger apoptosis. This is the first demonstration that a topically

applied compound can retard melanocytic lesion development by targeting a key signaling pathway that promotes melanoma.

Table 1

Table 1. IC_{50} of ISC-4 on normal, melanocytic lesion, and melanoma cell lines

IC_{50}, μmol/L	PBITC (Phenylbutyl isothiocyanate) 	ISC-4 (Phenylbutyl isoselenocyanate) 
Normal cells		
Human fibroblasts	37 ± 5	24 ± 3
Human mealanocytes	30 ± 5	19 ± 3
Melanoma cells		
WM35	9 ± 0.4	7 ±2
Sbcl2	10 ± 2	5 ± 0.4
UACC 903	17 ± 2	9 ± 0.9

ISC-4 kills melanocytic lesion and melanoma cells more effectively than normal skin cells. ISC-4 has been derived from naturally occurring isothiocyanates by increasing the alkyl carbon chain length to contain 4 carbons and replacing sulfur with selenium (structure shown in Table 1. ISC-4 can kill aggressive, invasive, advanced stage melanoma cells following systemic administration, but its effect on early melanocytic lesion and normal cells present in the skin is unknown. Human skin is composed of multiple cell types including fibroblasts, keratinocytes, and melanocytes, with the latter developing into noninvasive melanocytic lesions, which can progress into invasive melanoma. Therefore, effective topical chemopreventive agents would need to kill early noninvasive melanocytic lesion or invasive melanoma cells with negligible effect on normal skin cells. To determine the appropriate concentration range and IC50 of ISC-4 for topical use applications, cell viability using the MTS assay was examined after exposure of melanocytic lesion, melanoma, human epidermal melanocytes, or normal skin fibroblast cells to ISC-4 (Table 1). An ISC-4 concentration of 24 µmol/L was required to kill 50% of normal human fibroblast compared with 7 or 5.0 µmol/L for early-stage WM35 or Sbcl2 cells lines derived from an early-stage melanocytic lesion in the radial growth phase or 9 µmol/L for invasive UACC 903melanoma cells derived from an invasive cutaneous melanoma (Table 1). Thus, ISC-4 is 2- to 5-fold more effective at killing melanocytic lesion or melanoma compared with normal cells, indicating potential utility for topical applications at concentrations less than 19 µmol/L. PBITC served as a control to show the efficacy and importance of selenium in the structure of ISC-4.

ISC-4 decreases Akt3 activity and triggers apoptosis in melanocytic lesion cells derived from the radial growth phase and advanced stage melanoma cells.
To measure ISC-4 inhibition of Akt3 activity in early stage and advanced stage melanomas, WM35 or UACC 903 cells were exposed to 2.5 to 15 µmol/L of ISC-4 or control PBITC and cell lysates were analyzed by Western blotting.ISC-4 decreased pAkt3 levels at lower

concentrations than control PBITC, with negligible effect on total Akt protein levels (Fig. 6A). Similarly, a dose-dependent decrease in pAkt3 levels was also observed in UACC 903 cells (Fig. 6B).Furthermore, ISC-4 decreased levels of downstream pPRAS40 more effectively that control PBITC, which had little effect on this downstream signaling target. As a consequence of decreased Akt3 activity, cleaved PARP and caspase-3 indicating increased apoptosis rose more dramatically in ISC-4-treated than in PBITC-treated cells (Fig. 6A). Thus, ISC-4 functions to decrease Akt3 activity, resulting in increased apoptosis in radial growth phase melanocytic lesion and advanced stage melanoma cells.

siRNA-mediated inhibition of Akt3 retards melanoma tumor development by increasing apoptosis in melanoma cells. To show the effect of Akt3 knockdown on melanoma tumor development and cellular apoptosis levels, siRNA was used to inhibit protein expression. Western blot analysis confirmed knockdown of Akt3 protein levels and showed corresponding increases in cleaved caspase-3 levels, indicating an increase in levels of apoptosis (Fig. 6C). Decreasing Akt3 protein levels reduce tumor development by approximately 3-fold (Fig. 6D, $P < 0.01$;t test; left) by increasing cellular apoptosis by 6- to 7-fold (Fig. 6D, $P < 0.01$; t test; right). Thus, decreasing Akt3activity reduces tumor formation by increasing levels of cellular apoptosis.

Figure 6

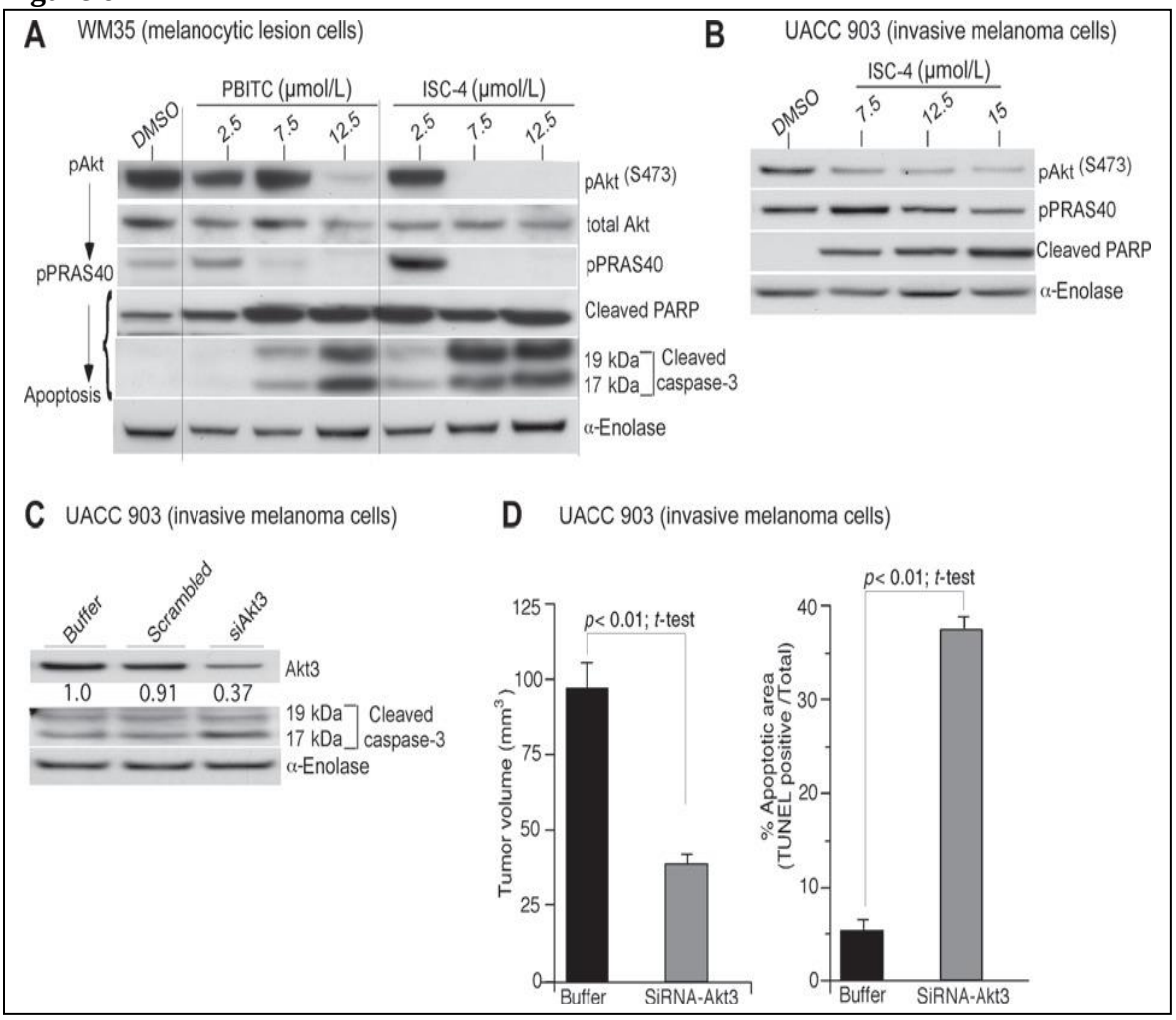


Figure 6. Targeting Akt3 using siRNA or ISC-4 induces apoptosis in cultured melanoma cells and melanoma tumor xenografts. A and B, ISC-4 inhibits Akt3 signaling in early melanocytic lesion cells and advanced stage melanoma cells. Dose-dependent decrease in pAkt and downstream pPRAS40 levels occurred with increasing ISC-4 concentration, leading to increased apoptosis observed as rising levels of cleaved PARP and caspase-3. C, siRNA-mediated knockdown of Akt3 increased levels of cleaved caspase-3. Increased level of cleaved caspase-3 (an apoptotic marker) was observed in cells in which Akt3 protein expression had been reduced using siRNA. D, siRNA-mediated inhibition of Akt3 reduced melanoma tumor development by increasing cellular apoptosis. Decreasing Akt3 protein levels led to a significant delay in tumor development on day 10 compared with control cells (left). Results of 3 independent experiments are plotted; bars, tumor size mean SEM. Rates of tumor cell apoptosis measured by TUNEL staining, increased with decreasing Akt3 protein levels (right).

Mechanism leading to death of cultured melanocytic lesion cells following ISC-4 treatment is by triggering apoptosis.

Mechanism decreasing cultured melanocyte, melanocytic lesion WM35, or advanced melanoma UACC 903 cell survival was established by measuring caspase3/7 activity following a 24-hour treatment protocol with ISC-4, PBITC, or vehicle control with MTS and Caspase 3/7 Assay kits(Fig. 7A and B). Both ISC-4 and PBITC decreased viability (Fig. 7A) by increasing apoptosis (Fig. 7B); however, ISC-4 had efficacy at lower concentrations. Thus, ISC-4 is effective at killing cultured melanocytic radial and vertical growth phase lesion cells with a lesser effect on normal human melanocytes (Fig. 7A and B; left).

Topical ISC-4 treatment inhibits melanocytic and melanoma lesion development in laboratory generated skin.

Human skin containing melanocytic lesions resembling benign for WM35 (Fig. 8A; left) or aggressive for UACC 903 (Fig. 8B; left) tumors seen in patient skin can be generated in the laboratory and effects of anticancer agents evaluated on lesion development in this model (33, 38). Both WM35and UACC 903 cell lines express GFPs, making melanocytic nodule development detectable and quantifiable by fluorescence microscopy (Fig. 8C and D; left). A decrease in lesion development was observed in laboratory-generated skin following ISC-4 treatment compared with controls (Fig. 8). H&E-stained and fluorescent images of cross-sections of skin at day 11 show dramatically fewer melanocytic lesion or melanoma cells in the skin than controls (Fig. 8A–C). In contrast to ISC-4–treated skin having little melanocytic lesion development, control skin contained nests of cells for the WM35-GFP cells line (Fig. 8A and C) and many invading disseminating cells for the UACC 903-GFP cell line (Fig. 8B and D). At the end of ISC-4 treatment, most melanocytic lesion cells for both cell lines were barely detectable (Fig. 8; right). ISC-4 also caused no detectable damage to the keratinocytes, fibroblasts, or skin morphology present in this model, again suggesting that a topical formulation would cause negligible effect on normal skin cells. To assess the effect of ISC-4 treatment on melanocytic lesion development overtime, serial measurement were made on the same skin (n = 3 skins with 10 photographs per skin, totaling 30 pictures) according to the treatment schedule shown in Figure 9A. Regression in area occupied by WM35-GFP and UACC 903-GFP lesions was observed when treated with ISC-4 compared with controls (Fig. 9B). A similar trend was observed for PBITC, but ISC-4 was more effective, leading to an 80% to 90% reduction in average area occupied compared with a 50% to 60%decrease for PBITC at the end of the treatment (Fig. 4C and D; $P < 0.001$). Thus, ISC-4 is effective at inhibiting melanocytic lesion development in the laboratory-generated skin model containing melanocytic lesions at concentrations ranging from 7.5 to 25 µmol/L, supporting topical use of ISC-4 for preventing melanocytic lesion development.

Figure 7

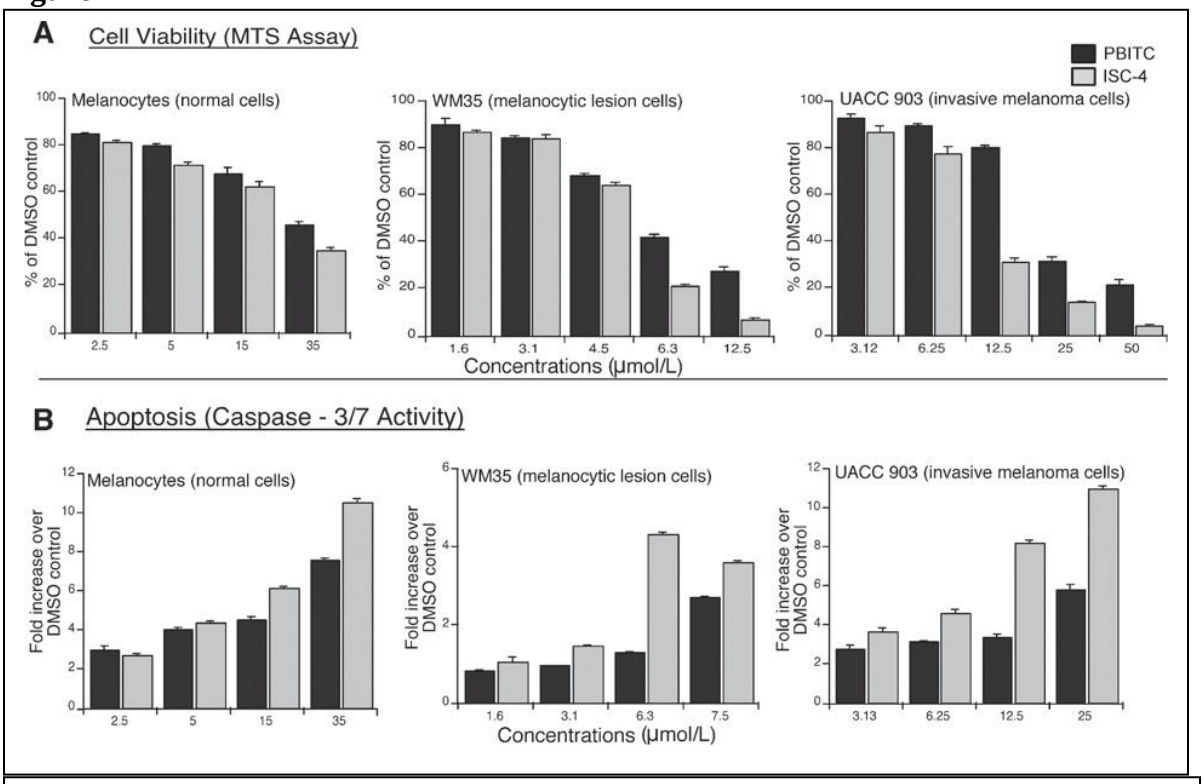


Figure 7. ISC-4 treatment inhibits survival of cultured melanoma cells and induces apoptosis. A, ISC-4 treatment decreased cell survival. Melanocytic lesion cells (WM35 and Sbcl2), advanced stage melanoma cells (UACCC 903), and normal epidermal human melanocytes were treated with increasing concentrations of PBITC or ISC-4 for 24 hours. Cell viability was measured by MTS assay. Results of 3 independent experiments were plotted; bars, mean SEM. B, ISC-4 treatment triggers apoptosis. PBITC or ISC-4 treatment for 24 hours increased caspase-3/7 activity in cell culture media. Bar graph, fold increase over DMSO-treated control. Results of 3 independent experiments were plotted; bars, mean +/- SEM.

Topical application of ISC-4 prevents melanocytic lesion development in the skin of nude mice with negligible changes in animal body weight.

To show that topical ISC-4 inhibits melanocytic lesion development in animals, UACC 903 melanoma cells that are tumorigenic and have high Akt3 signaling activity were injected subcutaneously, and 24 hours later, animals were treated topically everyday with ISC-4 or acetone vehicle (Fig. 10A). WM35 cells could not be evaluated in mice, as these cells do not grow or form detectable lesions in animals. Topical ISC-4 treatment led to a 77% decrease in UACC 903 tumor size compared with vehicle control (Fig. 10A; $P < 0.001$). Body weights of mice treated with ISC-4 compared with control showed no significant differences between groups, suggesting negligible toxicity (Fig. 10A; insert). Thus, the use of topical ISC-4 inhibited cutaneous melanocytic lesion development without weight loss that would indicate systemic toxicity.

Figure 8

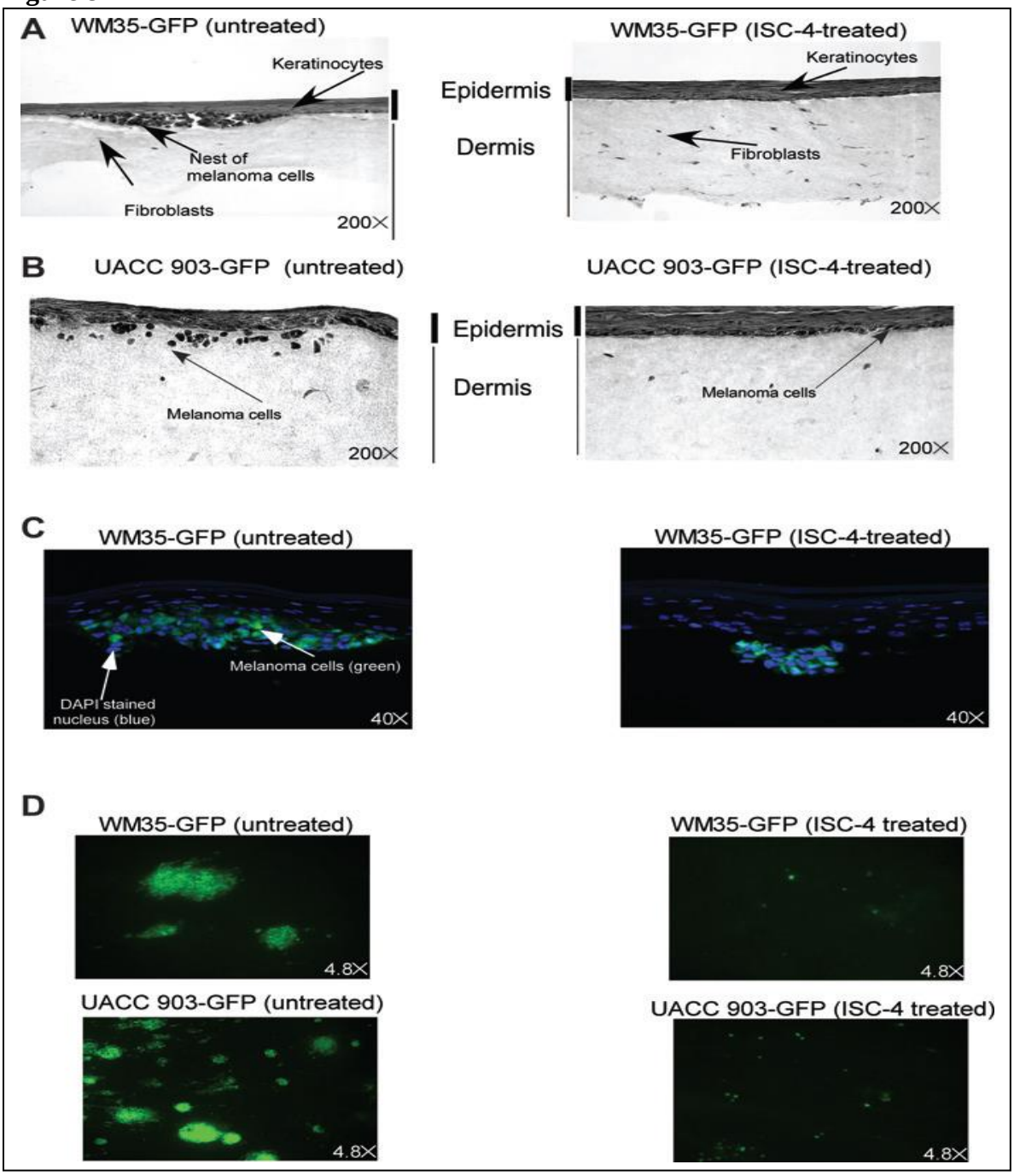

A
WM35-GFP (untreated)
Keratinocytes
Nest of melanoma cells
Fibroblasts
200×
WM35-GFP (ISC-4-treated)
Keratinocytes
Epidermis
Dermis
Fibroblasts
200×
B
UACC 903-GFP (untreated)
Melanoma cells
200×
UACC 903-GFP (ISC-4-treated)
Epidermis
Dermis
Melanoma cells
200×
C
WM35-GFP (untreated)
Melanoma cells (green)
DAPI stained nucleus (blue)
40×
WM35-GFP (ISC-4-treated)
40×
D
WM35-GFP (untreated)
4.8×
WM35-GFP (ISC-4 treated)
4.8×
UACC 903-GFP (untreated)
4.8×
UACC 903-GFP (ISC-4 treated)
4.8×

Figure 8. ISC-4 treatment reduced melanocytic lesion and melanoma development in laboratory generated skin. Skin was prepared containing radial growth phase melanocytic lesion WM35 or invasive UACC 903 melanoma cells and treated with ISC-4 or vehicle daily from days 6 to 11 at which point skin was processed for microscopy. For each treatment group, 3 skins were used. For each skin, 8 to 10pictures were taken daily, and the average areas occupied by the green nodules were quantified using the IP Labs image software. H&E-stained cross-section of the skin reconstructs at day 11containing: (A) WM35-GFP; or (B) UACC 903-GFP (200). C, fluorescence microscopy of frozen DAPI-stained cross sections of the skin containing GFP-tagged WM35-GFP (40). D, image is a fluorescent photomicrograph from the top of skin surface showing WM35-GFPand UACC 903-GFP untreated or treated with ISC-4 (40). Images show inhibition of cutaneous melanocytic lesion or invasive melanoma development followingISC-4 treatment.

Mechanistically, ISC-4 inhibits melanocytic lesion development in animals by inhibiting Akt3 activity to trigger tumor cell apoptosis.

To determine the mechanism that causes tumor inhibition, size- and time-matched tumors from mice treated with ISC-4 or vehicle were compared. Western blot analysis of matched tumors lysates harvested at day 13 from animals treated with ISC-4 showed decreased active pAkt ($P < 0.05$; t test) and downstream target pPRAS40 ($P < 0.001$;t test) compared with vehicle control–treated tumors (Fig. 5B). To show that decreased Akt3 activity led to an increase in tumor cell apoptosis, rates of apoptosis [TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) staining] and proliferation (Ki-67 immunohistochemistry)were compared in size- and time-matched melanoma tumors excised from ISC-4–treated animals and compared with vehicle control. Tumors harvested at days 11 and 13 from mice treated with ISC-4 showed approximately 3-fold (Fig. 5C; left, $P < 0.01$; t test) more TUNEL-positive cells than control animals treated with vehicle control. In contrast, no statistically significant difference was seen in rates of proliferation between different treatment groups (Fig. 5C; right). Thus, treatment of animals with ISC-4 decreased Akt3 signaling, leading to increased rates of tumor cells apoptosis.

ISC-4 caused negligible major organ-related toxicity.

To determine whether ISC-4 would cause systemic toxicity, blood parameters (serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, blood urea, glucose, and creatinine) indicative of organ toxicity were measured following systemic administration (Fig. 5D). None of these indicators were significantly different from controls. Furthermore, histologic examination of H&E-stained vital organ sections revealed that ISC-4 treatment did not significantly alter cell morphology or structure of liver, kidney, adrenal, lung, spleen, heart, pancreatic, or intestinal tissue. Thus, ISC-4 caused negligible systemic toxicity and would be effective for preventing melanocytic lesion development.

Figure 9

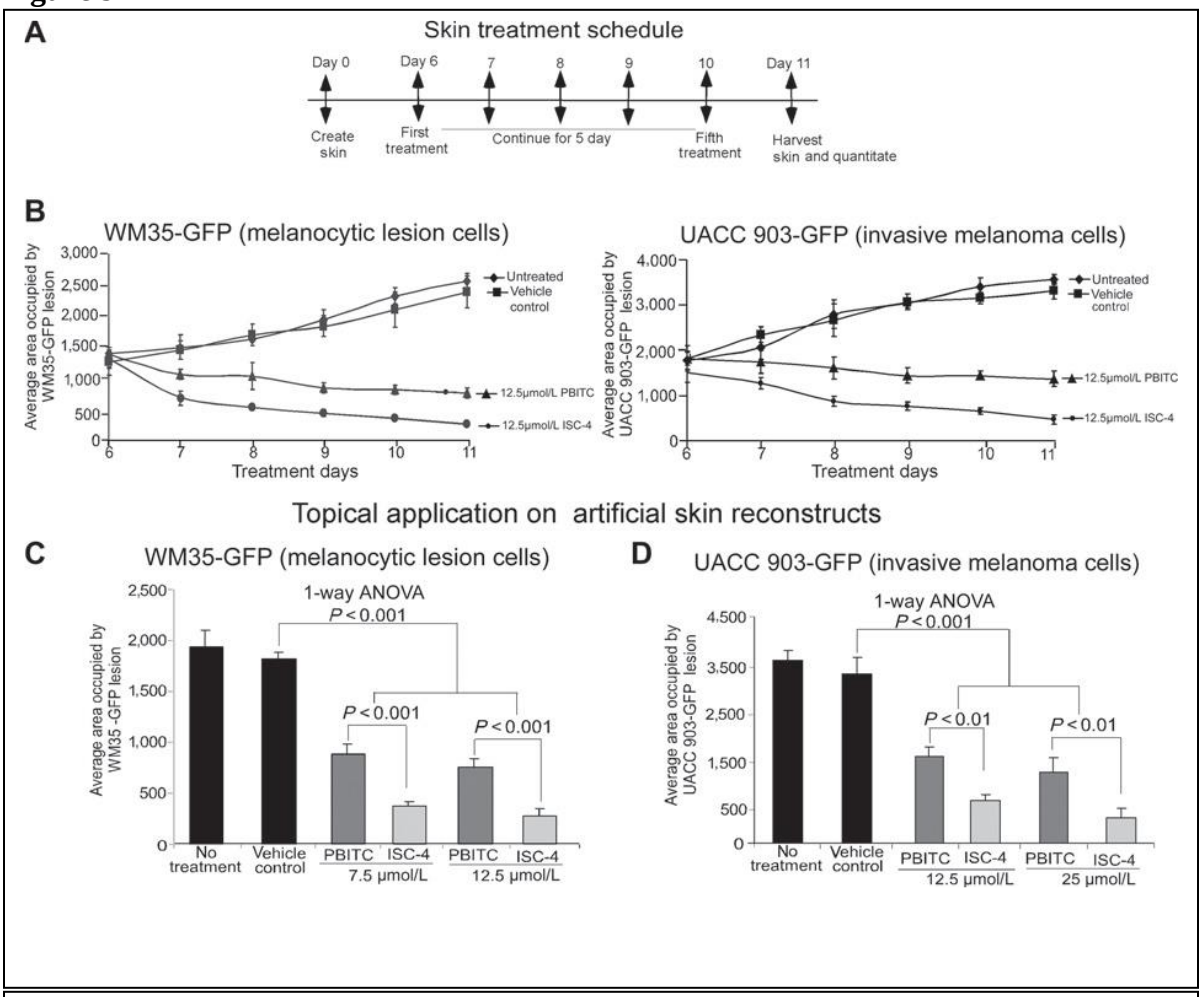


Figure 9. Melanocytic lesion or melanoma development following ISC-4 treatment in laboratory-generated skin. A, ISC-4 treatment regime. Laboratory generated skin at day 6 was treated daily with ISC-4, PBITC, or vehicle up to day 10. B, area occupied by GFP-tagged melanocytic lesions following ISC-4treatment. Area occupied by fluorescent melanocytic or melanoma cells in laboratory-generated skin was quantified daily by florescence microscopy and 2-dimensional image analysis software; values, mean +/- SE. (3 skins per group were used and for each skin 10 images were photographed). Daily treatment led to an 80% to 90% decrease in size by day 11. C, topical application of ISC-4 was more effective than PBITC at reducing melanocytic lesion development in laboratory-generated skin. UACC 903-GFP or WM35-GFP containing skin reconstructs were treated on day 6 with 7.5 to 25 μmol/L of ISC-4 or PBITC daily for 5 consecutive days and lesion development quantified and compared at day 11; values, mean +/_ SE. ISC-4 decreased lesion development 50% to 70% (P <0.001; 1-way ANOVA) more effectively than PBITC than controls.

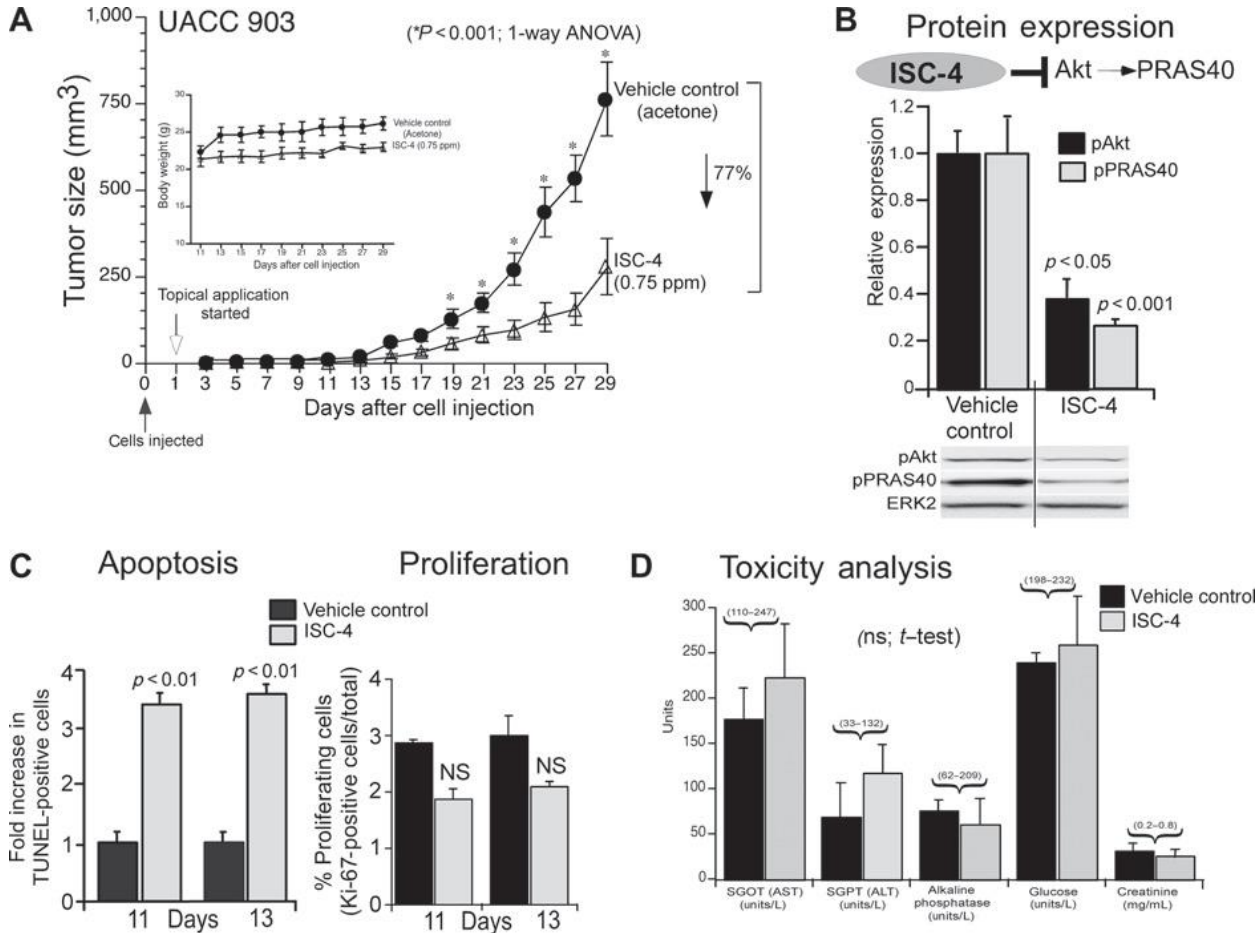


Figure 10. Topical ISC-4 retards cutaneous melanoma development by triggering apoptosis. A, ISC-4 inhibits cutaneous melanoma tumor growth in
animal skin. UACC 903 melanoma cells were subcutaneously injected into left and right flanks of 4 to 6 weeks old female athymic nude mice and 24 hours later
ISC-4 or acetone vehicle applied topically on each day. Tumors were measured on alternate days until day 29; values, mean _ SE. Topical application of ISC-4
led to a 77% decrease (P < 0.001) in tumor volume beginning from day 19 compared with acetone vehicle-treated control. No significant difference was
found between body weights of ISC-4- and acetone-treated animals (see inset). B, ISC-4 inhibits Akt3 signaling in cutaneous melanomas. Protein lysates from
ISC-4- or vehicle-treated, size- and time-matched tumors were harvested at day 13 for Western blot analysis; values, mean _ SE. ISC-4 decreased
pAkt (P < 0.05; t test) and downstream pPRAS40 (P < 0.001; t test) levels by 60% to 70% in ISC-4–treated tumor lysates compared with controls. C, ISC-4
increased tumor cell apoptosis but has a nonsignificant effect on tumor cell proliferative rates. Formalin-fixed, paraffin-embedded size- and time-matched
tumors sections from ISC-4- or vehicle-treated animals were subjected to TUNEL analysis for apoptosis or proliferation by Ki-67 staining; values, mean _ SE.
ISC-4 increased melanoma tumor cells apoptosis at days 11 and 13 compared with vehicle control (P < 0.01; 1-way ANOVA). No significant difference

was observed in tumor cell proliferation rates. D, ISC-4 caused negligible major organ-related toxicity in mice. Levels of serum glutamic oxaloacetic transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, glucose, and creatinine were analyzed in blood collected from animals treated with ISC-4 or vehicle control to measure effects on major organ-related toxicity. Values in brackets above the bars represent the normal rage of serum levels for nude mice. No significant differences were observed, indicating negligible vital organ related toxicity; values, mean _ SE.

A manuscript has been published. The manuscript was featured on the cover of the journal and discovery reported in the lay press on TV, radio and in print. The citation and abstract detailing the discovery are listed below:

Melanoma chemoprevention in skin reconstructs and mouse xenografts using isoselenocyanate-4. *Nguyen N, Sharma A, Nguyen N, Sharma AK, Desai D, Huh SJ, Amin S, Meyers C, Robertson GP. Cancer Prev Res (Phila). 2011 Feb;4(2):248-58.*

Abstract: Melanoma incidence and mortality rates continue to increase despite the use of sunscreen as well as screening programs for early surgical excision of premalignant lesions. The steady increase in melanoma incidence suggests that additional preventive approaches are needed to augment these existing strategies. One unexplored area involves targeting genes whose deregulation promotes disease development to prevent melanoma. The Akt3 signaling pathway is one key signaling cascade that plays a central role by deregulating apoptosis to promote development of approximately 70% of melanomas. Isoselenocyanate-4 (ISC-4), derived from isothiocyanates by increasing the alkyl chain length and replacing sulfur with selenium, has been developed to target this important signaling pathway in melanomas; however, its chemopreventive potential is unknown. In this study, the chemopreventive efficacy of topical ISC-4 was evaluated in a laboratory-generated human skin melanoma model containing early melanocytic lesion or advanced stage melanoma cell lines and in animals containing invasive xenografted human melanoma. Repeated topical application of ISC-4 reduced tumor cell expansion in the skin model by 80% to 90% and decreased tumor development in animals by approximately 80%. Histologic examination of ISC-4-treated skin showed no obvious damage to skin cells or skin morphology, and treated animals did not exhibit markers indicative of major organ-related toxicity. Mechanistically, ISC-4 prevented melanoma by decreasing Akt3 signaling that led to a 3-fold increase in apoptosis rates. Thus, topical ISC-4 can delay or slow down melanocytic lesion or melanoma development in preclinical models and could impact melanoma incidence rates if similar results are observed in humans.

The technology included in US Patent application - Application Number: 20080306148 (Development of ISC-4 Drugs for Inhibition of AKT Pathway Signaling in Cancer [***Anti-Cancer Compositions & Methods (Novel ISC Drugs***)]), described above was also, on 04/19/2012, successfully licensed to Melanovus Oncology for further development through a phase I clinical trial.

A SBIR grant in which Penn State is a significant subcontractor was submitted but not funded in collaboration with PenForGel, LLC. These funds would enable the topical agent to be tested in patients in a clinical trial that would take place at Penn State University.

The *objective of this SBIR proposal was* to develop novel topical clinical formulations of ISC-4 and measure loading efficiency, agent stability, and permeation across organotypic and human skin. Efficacy for inhibiting or decreasing melanocytic lesion development will be tested in laboratory generated skin. Substantial preliminary data developed by PenForGel, LLC demonstrates the potential feasibility of this novel topical agent for preventing melanocytic lesion development using an experimental preclinical topical formulation. It is unlikely that this agent would be used for treating patients with advanced stage melanoma having widely disseminated tumors in distant organs, but rather for early or ongoing management, or possible prevention of melanoma. The *central hypothesis* for these studies is that targeting Akt3 signaling using a novel topical formulation containing ISC-4 would be an effective targeted approach for managing or preventing melanoma development, or for treating unresectable lesions in skin for which surgery is not an option. This hypothesis will be tested and the objectives of the research accomplished by: (1) Development of a topical formulation of ISC-4 for clinical evaluation; and (2) Establishing the inhibitory efficacy of the topical clinical formulation for preventing melanocytic lesion development in laboratory generated human skin containing tumors that resemble early melanomas. Collectively, these discoveries are expected to form a crucial portion of the foundation for an application by PenForGel, LLC to the FDA to obtain IND status for topical ISC-4 to enable evaluation in a phase I clinical trial. ISC-4 for topical applications has been patented and a pre-IND meeting has been arranged with the FDA to discuss the plan for the clinical evaluation and trial in which topical ISC-4 would be tested in humans. Dr. Joseph Drabick, M. D. at Penn State would serve as the PI on the clinical trial.

Significant progress has been made on this project. Specific accomplishments are:

1. An HPLC / LC-MS assay has been developed for measuring the concentration of ISC-4 in media, in skin layers and in the serum of animals. This assay is now being used to assess the loading and stability of ISC-4 in a variety of topical formulations that have also been developed over the last year.
2. A potential viable clinical topical formulation was developed from a number of formulations over the last year. It has been tested on mouse and human skin and found to deliver ISC-4 into the skin. The HPLC / LC-MS assays also developed last year are being used to assess the amount and stability of the ISC-4 delivery into the skin. The next goal is to test this potential clinical topical formulation on animals and determine whether it causes toxicity.
3. An SBIR grant focusing on topical formulation development was submitted in collaboration with PenForGel, LLC. The application was reviewed and a revised application submitted but not funded.
4. The intellectual property related to ISC-4 was licensed to Melanovus Oncology in the first quarter of 2012 for commercialization. The company is pursuing the clinical development of these agents and is in the process of arranging a pre-IND meeting with the FDA. At this stage, Penn State has little involvement in this process since we do not

work for this company and Penn State's conflict of interest team led by Don Martin has explicitly stated that we must not use research funds to support the development of agents that would benefit the company. To my knowledge, the company is currently seeking investors to support the clinical development of the compound.

5. Since the licensing of ISC-4 to Melanovus Oncology and subsequent potential for a conflict of interest, funds were approved to purchase a laser to slightly extend the original studies and reduce the potential for conflict of interest arising from the licensing to Melanovus Oncology. The remaining studies prevent the conflict by developing thermoablative particles that could be combined with ISC-4 to synergistically kill melanoma cells. Although this project is related to the original Aims of the project it does not overlap with the objectives of Melanovus Oncology to test the agent's toxicity and pharmacokinetics in other animal models.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving

Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
____Yes
____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
Melanoma chemoprevention in skin reconstructs and mouse xenografts using isoselenocyanate-4.	*Nguyen N, Sharma A, Nguyen N, Sharma AK, Desai D, Huh SJ, Amin S, Meyers C, Robertson GP.*	Cancer Prevention Research	May 2010	☐Submitted ☐Accepted X Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____ No____X_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The technology included in US Patent application - Application Number: 20080306148 (Development of ISC-4 Drugs for Inhibition of AKT Pathway Signaling in Cancer [***Anti-Cancer Compositions & Methods (Novel ISC Drugs***)]), described above was, on 04/19/2012, successfully licensed to Melanovus Oncology for further development through a phase I clinical trial.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes ___ No ___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

Yes_____ No_____

If yes, indicate number of patent, title and date issued:

Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No ___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Robertson, Gavin P.	POSITION TITLE Professor
eRA COMMONS USER NAME (credential, e.g., agency login) gprobertson	

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
California State University, Northridge, CA	B.A.	06/88	Molecular Biology
University of California, Riverside, CA	Ph.D.	03/97	Biomedical Sciences
Ludwig Institute for Cancer Research, San Diego, CA	Postdoc	03/97-03/00	Cancer Research

A. Personal Statement

Dr. Robertson served as the Project Principal Investigator.

B. Positions and Honors

Positions and Employment

1988 Student Assistant in Genetics, California State University, Northridge, CA
1989-1991 Coordinator of "Student Panels for an International Curriculum and Education" at Office of International and Exchange Programs, California State University, Northridge, CA
1987-1989 Drosophilla Stock Technician, California State University, Northridge, CA
1989-1991 Teaching Assistant in Cell Biology, California State University, Northridge, CA
1990-1991 Teaching Assistant in Embryology, California State University, Northridge, CA
1990-1992 Cytogenetic Technologist at Kaiser Permanente Regional Genetics Laboratory, CA
1992-1997 Research Assistant at University of California, Riverside, CA
1997-2000 Postdoctoral Fellow at Ludwig Institute for Cancer Research, San Diego, CA
2000-2006 Assistant Professor (tenure track), Departments of Pharmacology, Pathology, and Dermatology, Penn State University College of Medicine, Hershey, PA
2006-2008 Co-Director, Drug Development and Discovery Core, Penn State University College of Medicine, Hershey, PA
2006-2010 Associate Professor (with tenure), Departments of Pharmacology, Pathology, and Dermatology, Penn State University College of Medicine, Hershey, PA
2008-2010 Associate Director for Translational Research, Penn State Cancer Institute, Penn State University, College of Medicine, Hershey, PA
2006-2011 Director, Experimental Therapeutics Program, Penn State Cancer Institute, Penn State University College of Medicine, Hershey, PA
2005-present Director, Melanoma Therapeutics Program, Department of Pharmacology, Penn State University College of Medicine, Hershey, PA
2010-present Director, Melanoma Center, Department of Pharmacology, Penn State University College of Medicine, Hershey, PA

2010-present Professor, Departments of Pharmacology, Pathology, Dermatology and Surgery, Penn State University College of Medicine, Hershey, PA

California State University, Northridge (CSUN) Associated Students Grant
California State University, Northridge (CSUN) Biology Department Grant
Graduate Student Support Grant
California State University, Northridge (CSUN) Foundation Grant
Northridge Hospital Grant
Kaiser Permanente Scholarship
Sigma XI Scientific Research Society Award for Graduate Research in Biology
University of California at Riverside Graduate Student Council Minigrant Award
Amgen Summer Fellowship
Travel Award from the University of California at Riverside Division of Biomedical Sciences
University of California at Riverside Dissertation Research Grant
Sigma XI Research Award
Joy K. Tilton Cancer Research Award
NIH/NCI Cancer Training Grants (CCT0198, CCT0197 & 33150A)
American Cancer Society Research Scholar (2004)

C. Peer-Reviewed Publications (in chronological order)

1. Robertson G, Coleman A, Lugo TG. A malignant melanoma tumor suppressor on human chromosome 11. Cancer Res. 1996; 56(19):4487-92.
2. Robertson GP, Hufford A, Lugo TG. A panel of transferable fragments of human chromosome 11q. Cytogenet Cell Genet. 1997; 79(1-2):53-59.
3. Robertson GP, Furnari FB, Miele ME, Glendening MJ, Welch DR, Fountain JW, Lugo TG, Huang HJ, Cavenee WK. In vitro loss of heterozygosity targets the PTEN/MMAC1 gene in melanoma. Proc Natl Acad Sci USA. 1998; 95(16): 9418-23. PMCID: PMC21353
4. Robertson GP, Lee V, Bucher M, Shulkin J, Jamehdor M, Lugo TG. Use of G-banded karyotypes to estimate the physical size or marker chromosomes. The Journal of the Association of Genetic Technologists 1998; 24:201-4.
5. Robertson GP, Goldberg EK, Lugo TG, Fountain JW. Functional localization of a melanoma tumor suppressor gene to a small (< or = 2 Mb) region on 11q23. Oncogene. 1999;18(20):3173-80.
6. Robertson GP, Herbst RA, Nagane M, Huang HJ, Cavenee WK. The chromosome 10 monosomy common in human melanomas results from loss of two separate tumor suppressor loci. Cancer Res. 1999; 59(15):3596-601.
7. Robertson GP, Huang HJ, Cavenee WK. Identification and validation of tumor suppressor genes. Mol Cell Biol Res Commun. 1999; 2(1):1-10.
8. Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Su Huang H, Petrelli NJ, Zhang X, O'Dorisio MS, Held WA, Cavenee WK, Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000; 24(2):132-8.
9. Robertson GP, Huang H-JS, Cavenee WK. Loss of hetreozygosity (LOH). In John Wiley & Sons L, ed. Wiley Encyclopedia of Molecular Medicine, Vol. 5, 2002. pp. 1959-62.
10. Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA. Suppression of BRAF(V599E) in human melanoma abrogates transformation. Cancer Res 2003; 63(17):5198-202.
11. Robertson GP, Sargent L, Nelson MA. Positional approaches to cancer genetics. Methods in molecular biology 2003; 222:339-64.

12. Stahl JM, Cheung M, Sharma A, Trivedi NR, Shanmugam S, Robertson GP. Loss of PTEN promotes tumor development in malignant melanoma. Cancer Res 2003; 63(11):2881-90.
13. Cheung M, Miller C, Robertson GP. BRaf and malignant melanoma. Recent Research Developments in Cancer, Vol. 6, 2004. pp. 1-19.
14. Karbowniczek M, Cash T, Cheung M, Robertson GP, Astrinidis A, Henske EP. Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. J Biol Chem 2004; 279(29):29930-7.
15. Miller CJ, Cheung M, Sharma A, Clarke L, Helm K, Mauger D, Robertson GP. Method of mutation analysis may contribute to discrepancies in reports of (V599E)BRAF mutation frequencies in melanocytic neoplasms. J Invest Dermatol 2004; 123(5):990-2.
16. Stahl JM, Sharma A, Cheung M, Zimmerman M, Cheng JQ, Bosenberg MW, Kester M, Sandirasegarane L, Robertson GP. Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res 2004; 64(19):7002-10.
17. Wang S, Robertson GP, Zhu J. A novel human homologue of Drosophila polycomblike gene is up-regulated in multiple cancers. Gene 2004; 343(1):69-78.
18. Croushore JA, Blasiole B, Riddle RC, Thisse C, Thisse B, Canfield VA, Robertson GP, Cheng KC, Levenson R. Ptena and ptenb genes play distinct roles in zebrafish embryogenesis. Dev Dyn 2005; 234(4):911-21. PMCID: PMC2611959
19. Robertson GP. Functional and therapeutic significance of Akt deregulation in malignant melanoma. Cancer metastasis reviews 2005; 24(2):273-85.
20. Sharma A, Trivedi NR, Zimmerman MA, Tuveson DA, Smith CD, Robertson GP. Mutant V599EB-Raf regulates growth and vascular development of malignant melanoma tumors. Cancer Res 2005; 65(6):2412-21.
21. Stover TC, Sharma A, Robertson GP, Kester M. Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. Clin Cancer Res 2005; 11(9):3465-74.
22. Karbowniczek M, Robertson GP, Henske EP. Rheb inhibits C-raf activity and B-raf/C-raf heterodimerization. J Biol Chem 2006; 281(35):25447-56.
23. Sharma A, Tran MA, Liang S, Sharma AK, Amin S, Smith CD, Dong C, Robertson GP. Targeting mitogen-activated protein kinase/extracellular signal-regulated kinase kinase in the mutant (V600E) B-Raf signaling cascade effectively inhibits melanoma lung metastases. Cancer Res 2006; 66(16):8200-9. PMCID: PMC2777627
24. Liang S, Sharma A, Peng HH, Robertson G, Dong C. Targeting mutant (V600E) B-Raf in melanoma interrupts immunoediting of leukocyte functions and melanoma extravasation. Cancer Res 2007; 67(12):5814-20. PMCID: PMC2724629
25. Madhunapantula SV, Sharma A, Robertson GP. PRAS40 deregulates apoptosis in malignant melanoma. Cancer Res 2007; 67(8):3626-36.
26. Robertson GP. Mig-7 linked to vasculogenic mimicry. Am J Pathol 2007; 170(5):1454-6. PMC1854941
27. Sharma A, Robertson GP. Models of Melanoma Metastasis: Using a Transient siRNA-based Protein Inhibition Strategy in Mice to Validate the Functional Relevance of Pharmacological Agents. Current Protocols in Pharmacology, 2007. pp. 14.6.1-14.6.15.
28. Cheung M, Sharma A, Madhunapantula SV, Robertson GP. Akt3 and mutant V600E B-Raf cooperate to promote early melanoma development. Cancer Res 2008; 68(9):3429-39. PMCID: PMC2603082
29. Girroir EE, Hollingshead HE, Billin AN, Willson TM, Robertson GP, Sharma AK, Amin S, Gonzalez FJ, Peters JM. Peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) ligands inhibit growth of UACC903 and MCF7 human cancer cell lines. Toxicology 2008; 243(1-2):236-43. PMCID: PMC2278023

30. Kester M, Heakal Y, Fox T, Sharma A, Robertson GP, Morgan TT, Altinoglu EI, Tabakovic A, Parette MR, Rouse SM, Ruiz-Velasco V, Adair JH. Calcium phosphate nanocomposite particles for in vitro imaging and encapsulated chemotherapeutic drug delivery to cancer cells. Nano Lett 2008; 8(12):4116-21.
31. Lung HL, Lo CC, Wong CC, Cheung AK, Cheong KF, Wong N, Kwong FM, Chan KC, Law EW, Tsao SW, Chua D, Sham JS, Cheng Y, Stanbridge EJ, Robertson GP, Lung ML. Identification of tumor suppressive activity by irradiation microcell-mediated chromosome transfer and involvement of alpha B-crystallin in nasopharyngeal carcinoma. Int J Cancer 2008; 122(6):1288-96.
32. Madhunapantula SV, Desai D, Sharma A, Huh SJ, Amin S, Robertson GP. PBISe, a novel selenium-containing drug for the treatment of malignant melanoma. Mol Cancer Ther 2008; 7(5):1297-308.
33. Madhunapantula SV, Robertson GP. Is B-Raf a good therapeutic target for melanoma and other malignancies? Cancer Res 2008; 68(1):5-8.
34. Sharma AK, Sharma A, Desai D, Madhunapantula SV, Huh SJ, Robertson GP, Amin S. Synthesis and anticancer activity comparison of phenylalkyl isoselenocyanates with corresponding naturally occurring and synthetic isothiocyanates. J Med Chem 2008; 51(24):7820-6. PMCID: PMC2629797
35. Tran MA, Gowda R, Sharma A, Park EJ, Adair J, Kester M, Smith NB, Robertson GP. Targeting V600EB-Raf and Akt3 using nanoliposomal-small interfering RNA inhibits cutaneous melanocytic lesion development. Cancer Res 2008; 68(18):7638-49.
36. Tran MA, Smith CD, Kester M, Robertson GP. Combining nanoliposomal ceramide with sorafenib synergistically inhibits melanoma and breast cancer cell survival to decrease tumor development. Clin Cancer Res 2008; 14(11):3571-81.
37. Dong C, Robertson GP. Immunoediting of leukocyte functions within the tumor microenvironment promotes cancer metastasis development. Biorheology 2009; 46(4):265-79. PMCID: PMC2777629
38. Gaitonde S, De SK, Tcherpakov M, Dewing A, Yuan H, Riel-Mehan M, Krajewski S, Robertson G, Pellecchia M, Ronai Z. BI-69A11-mediated inhibition of AKT leads to effective regression of xenograft melanoma. Pigment Cell Melanoma Res 2009; 22(2):187-95. PMCID: PMC2860277
39. Madhunapantula SV, Robertson GP. The PTEN-AKT3 signaling cascade as a therapeutic target in melanoma. Pigment Cell Melanoma Res 2009; 22(4):400-19.
40. Mounir Z, Krishnamoorthy JL, Robertson GP, Scheuner D, Kaufman RJ, Georgescu MM, Koromilas AE. Tumor suppression by PTEN requires the activation of the PKR-eIF2alpha phosphorylation pathway. Science signaling 2009; 2(102):ra85.
41. Salli U, Fox TE, Carkaci-Salli N, Sharma A, Robertson GP, Kester M, Vrana KE. Propagation of undifferentiated human embryonic stem cells with nano-liposomal ceramide. Stem Cells Dev 2009; 18(1):55-65.
42. Sharma A, Sharma AK, Madhunapantula SV, Desai D, Huh SJ, Mosca P, Amin S, Robertson GP. Targeting Akt3 signaling in malignant melanoma using isoselenocyanates. Clin Cancer Res 2009; 15(5):1674-85. PMCID: PMC2766355
43. Tran MA, Watts RJ, Robertson GP. Use of liposomes as drug delivery vehicles for treatment of melanoma. Pigment Cell Melanoma Res 2009; 22(4):388-99. PMCID: PMC2770731
44. Desai D, Madhunapantula SV, Gowdahalli K, Sharma A, Chandagaludoreswamy R, El-Bayoumy K, Robertson GP, Amin S. Synthesis and characterization of a novel iNOS/Akt inhibitor Se,Se'-1,4-phenylenebis(1,2-ethanediyl)bisisoselenourea (PBISe)--against colon cancer. Bioorg Med Chem Lett 2010; 20(6):2038-43. PMCID: PMC2892985

45. Huh SJ, Chen YL, Friedman SL, Liao J, Huang HJ, Cavenee WK, Robertson GP. KLF6 Gene and early melanoma development in a collagen I-rich extracellular environment. J Natl Cancer Inst 2010; 102(15):1131-47. PMCID: PMC2914760
46. Huh SJ, Chung CY, Sharma A, Robertson GP. Macrophage inhibitory cytokine-1 regulates melanoma vascular development. Am J Pathol 2010; 176(6):2948-57. PMCID: PMC2877855
47. Huh SJ, Liang S, Sharma A, Dong C, Robertson GP. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer Res 2010; 70(14):6071-82. PMCID: PMC2905495
48. Inamdar GS, Madhunapantula SV, Robertson GP. Targeting the MAPK pathway in melanoma: why some approaches succeed and other fail. Biochem Pharmacol 2010; 80(5):624-37. PMCID: PMC2897908
49. Madhunapantula SV, Mosca P, Robertson GP. Steroid hormones drive cancer development. Cancer Biol Ther 2010; 10(8).
50. Madhunapantula SV, Gowda R, Inamdar GS, Robertson GP. In situ photoimmunotherapy: a new hope for cutaneous melanoma patients. Cancer Biol Ther 2010; 10(11):1088-90.
51. Mathew R, Jia W, Sharma A, Zhao Y, Clarke LE, Cheng X, Wang H, Salli U, Vrana KE, Robertson GP, Zhu J, Wang S. Robust activation of the human but not mouse telomerase gene during the induction of pluripotency. FASEB J 2010; 24(8):2702-15. PMCID: PMC2909285
52. Sudol M, Tran M, Nowak MG, Flanagan JM, Robertson GP, Katzman M. A nonradioactive plate-based assay for stimulators of nonspecific DNA nicking by HIV-1 integrase and other nucleases. Anal Biochem 2010; 396(2):223-30. PMCID: PMC2787845
53. Cheng Y, Ren X, Zhang Y, Patel R, Sharma A, Wu H, Robertson GP, Yan L, Rubin E, Yang JM. eEF-2 kinase dictates cross-talk between autophagy and apoptosis induced by Akt Inhibition, thereby modulating cytotoxicity of novel Akt inhibitor MK-2206. Cancer Res 2011; 71(7):2654-63. PMCID: PMC3210447
54. Chung CY, Madhunapantula SV, Desai D, Amin S, Robertson GP. Melanoma prevention using topical PBISe. Cancer Prev Res (Phila) 2011; 4(6):935-48.
55. Madhunapantula SV, Robertson GP. Therapeutic Implications of Targeting AKT Signaling in Melanoma. Enzyme Res 2011; 2011:327923. PMCID: PMC3065045
56. Madhunapantula SV, Mosca PJ, Robertson GP. The Akt signaling pathway: An emerging therapeutic target in malignant melanoma. Cancer Biol Ther 2011; 12(12). PMCID: PMC3335938
57. Mosca PJ, Robertson GP. Augmentation of tumor-specific immunity by upregulation of apoptotic melanoma cell calreticulin expression. Cancer Biol Ther 2011; 11(6):581-3.
58. Nguyen N, Sharma A, Sharma AK, Desai D, Huh SJ, Amin S, Meyers C, Robertson GP. Melanoma chemoprevention in skin reconstructs and mouse xenografts using isoselenocyanate-4. Cancer Prev Res (Phila) 2011; 4(2):248-58. PMCID: PMC3210697
59. Sudol M, Fritz JL, Tran M, Robertson GP, Ealy JB, Katzman M. Evaluation of a system to screen for stimulators of non-specific DNA nicking by HIV-1 integrase: application to a library of 50,000 compounds. Antivir Chem Chemother 2011; 22(2):67-74.
60. Zhang P, Ozdemir T, Chung CY, Robertson GP, Dong C. Sequential binding of alphaVbeta3 and ICAM-1 determines fibrin-mediated melanoma capture and stable adhesion to CD11b/CD18 on neutrophils. J Immunol 2011; 186(1):242-54. PMCID: PMC3058329
61. Gowda R, Madhunapantula SV, Desai D, Amin S, Robertson GP. Selenium-containing histone deacetylase inhibitors for melanoma management. Cancer Biol Ther. 2012;13(9). PMCID:
PMC3399702.

62. Madhunapantula SV, Hengst J, Gowda R, Fox TE, Yun JK, Robertson GP. Targeting sphingosine kinase-1 to inhibit melanoma. Pigment Cell Melanoma Res. 2012;25(2):259-74. PMCID: PMC3290340.
63. Madhunapantula SV, Robertson GP. Chemoprevention of melanoma. Adv Pharmacol. 2012;65:361-98. PubMed PMID: 22959032. Need PMCID
64. Sharma A, Madhunapantula SV, Robertson GP. Toxicological considerations when creating nanoparticle-based drugs and drug delivery systems. Expert Opin Drug Metab Toxicol 2012; 8(1):47-69. PMCID: PMC3245366.
65. Singh S, Sharma A, Robertson GP. Realizing the clinical potential of cancer nanotechnology by minimizing toxicologic and targeted delivery concerns. Cancer Res. 2012 Nov 15;72(22):5663-8. Epub 2012 Nov 8. PubMed PMID: 23139207 Need PMCID
66. Gowda R, Madhunapantula SV, Desai D, Amin S, Robertson GP. Simultaneous Targeting of COX-2 and AKT Using Selenocoxib-1-GSH to Inhibit Melanoma. Mol Cancer Ther. 2013 Jan 3. [Epub ahead of print]. PubMed PMID: 23112250 Need PMCID

D. Research Support

Ongoing Research Support

No Number (Robertson) 01/01/04-12/31/15
Foreman Foundation

Melanoma Therapeutics Program Development
The goal of this project is to development of a Melanoma Therapeutics Program at Penn State.

5 R01 CA127892-05 (Robertson, Gavin P.) 12/17/2007-11/30/2013
NIH/NCI
"Akt3 Signaling as a Therapeutic Target"
The goal of this project is to develop novel agents that inhibit Akt3 signaling in melanomas and synergize with agents inhibiting other signaling cascades.

5 R01 CA138634-04 (Robertson, Gavin P.) 1/1/2010-12/31/2014
NIH/NCI
"Synergistically Acting Targeted Therapeutics for Melanoma"
Our long-term goal is to develop therapeutic agents that inhibit proteins deregulated in melanoma leading to synergistically acting tumor inhibition.

R01 CA136667-04 (Robertson, Gavin P.) 3/12/2010-12/31/2014
NIH/NCI
"Targeted Chemoprevention for Melanoma"
The objectives of this application are to first, evaluate the chemopreventive efficacy of novel nanoliposomes containing siRNA-targeting Akt3 and mutant V600EB-Raf, GSK3 or Wee1 kinases in a transgenic animal model of the disease in which both Akt3 and V600EB-Raf are deregulated to promote spontaneous melanocytic lesion development. Second, we will determine whether these agents can prevent or decrease lymph node invasion by early melanocytic cells to decreased disease development and aid survival.

1 R43 CA165610-01A1 (Robertson, Gavin P.) 9/26/2012-3/25/2013
NIH/PenForGel
"Establishing Bioactive Serum and Toxicity Levels for Nanolipolee 007"

Studies outlined in this proposal are to establish a method to measure leelamine levels in serum and tissue following administration of the agent to determine levels necessary for tumor inhibitory efficacy, dosing and identify levels leading to toxicity.

Completed Research Support (past 3 years)

5 R03 CA128033-02 (Robertson, Gavin P.) 9/1/2007-8/31/2010
NIH/NCI
"Targeted Chemoprevention through Inhibition of Akt3 Signaling"
The goal of this project is to characterize the chemopreventive potential of novel isothiocyanate derivatives that are effective for inhibiting Akt3 signaling.

Grant (Goldenberg, David/Robertson, Gavin P.) 4/20/2009-6/30/2011
PA Tobacco Settlement Fund/Dean's Feasibility
"P16 Alteration and BRAF Mutation and Patient Outcomes in Papillary Thyroid Cancer"
The aims of this project are: 1) To analyze surgical specimens of Papillary Thyroid Cancer (PTC) for the presence of p16 alterations and BRAF mutations. 2) To correlate the concurrent presence of p16 alterations and BRAF mutations with patient outcomes as manifested by distant metastases, local recurrence and loss of iodine activity.

#410047645 (Robertson, Gavin P.) 7/8/2009-6/30/2010
PA Tobacco Settlement Fund/Research Start-Up
"Synergistically Acting Targeted Therapeutics for Melanoma"
The goal of the Research Start-up Grant will be to obtain additional preliminary data supporting the studies proposed in the R01 submission. Our long-term goal is to develop therapeutic agents that inhibit proteins deregulated in melanoma leading to synergistically acting tumor inhibition.

SAP #4100047645 (Robertson, Gavin P.) 4/30/2009-12/31/2012
PA Tobacco Settlement Funds
"Moving Experimental Cancer Therapeutics from the Research Bench to the Clinic"
Our long-term goal is to develop an agent that could be used clinically to target the Akt3 signaling cascade and thereby effectively inhibit melanoma development. The objective of this application is to undertake the necessary characterization to move ISC-4, an agent we have identified inhibits the Akt3 signaling cascade, from the preclinical arena to the clinic.

SAP# 4100050904 (Connor, James R.) 4/21/2010-12/31/2012
PA Tobacco Settlement
"Multifunctional Nanoparticles for Melanoma and Brain Cancer"
The goal of this project will be to develop preliminary research data that will support a program project application.