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

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Grant support: This work was supported by The American Cancer Society [RSG-04-053-01-GMC], NIH [CA-127892-01A], The Foreman Foundation for Melanoma Research (to G. P. Robertson); NIH contract NO2-CB-56603 (to S. Amin); Elsa U. Pardee Foundation (to Arati Sharma and A K. Sharma), Melanoma Research Foundation with support in part from the Mike Geltrude Foundation and PA Department of Health (to Arati Sharma).

Running title: Topical ISC-4 for melanoma prevention

Key words: cutaneous melanoma, topical treatment, isothiocyanates, isoselenocyanates, Akt3.

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ABSTRACT

Melanoma incidence and mortality rates continue to rise 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 in order to prevent melanoma. The Akt3 signaling pathway is one key signaling cascade playing a central role by deregulating apoptosis to promote development of ~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 as well as in animals containing invasive xenografted human melanoma. Repeated topical application of ISC-4 reduced tumor cell expansion 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 delay or slow melanocytic lesion or melanoma development in preclinical models and could impact melanoma incidence rates if similar results are observed in humans.
INTRODUCTION

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 (1-4). Dacarbazine, which is nonspecific DNA alkylating agent, is an approved chemotherapeutics for advanced stage melanoma but is relatively ineffective at treating melanoma (5-7). This is equally true of other available therapeutic strategies for metastatic melanoma including immuno-, radio- and chemotherapy (8, 9). While surgical excision can eliminate melanomas if lesions are removed at the earliest stages of the disease, a significant number are not being detected or excised early, leading to rising incidence and mortality rates (9). 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 MAP kinase pathway activity and also by deregulating apoptosis (10, 11). Although three mammalian isoforms of Akt have been identified called Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ (12, 13), Akt3 is the predominantly active isoform in early melanocytic lesions and advanced melanomas, (14). Akt3 has a very low mutation rate and is preferentially activated in ~70% of melanoma patients due to increases in gene copy number and/or loss of a negative regulatory phosphatase called PTEN (14, 15). Increased Akt3 activity promotes melanocytic lesion development by decreasing V600E-B-Raf activity in the MAP kinase pathway and by decreasing the apoptotic sensitivity of melanoma cells, mediated through caspase-3 (11, 14). 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 Akt3 signaling in melanoma cells from a screen of natural products, which inhibited this pathway (16). Isothiocyanates are naturally occurring compounds found in cruciferous vegetables having anti-cancer properties (17-19), protecting against murine tumorigenesis induced by environmental carcinogens such as polycyclic aromatic hydrocarbons and nitrosamines (20, 21). Certain studies suggest isothiocyanates can act by
inhibiting the PI3 kinase pathway (22, 23). Unfortunately, isothiocyanates had low chemotherapeutic potency on melanoma cells requiring high concentrations for therapeutic efficacy, which made these compounds unsuitable therapeutic or preventive agents (16, 24). To overcome this limitation, more potent analogs called isoselenocyanates were developed using the isothiocyanate backbone but increasing the alkyl chain length and replacing sulfur with selenium (16, 24). Selenium was incorporated into the structure since it can be an effective chemopreventive agent (25-27) and deficiency frequently occurs in cancer patients including those diagnosed with metastatic melanoma (28). Isoselenocyanates had improved therapeutic efficacy for killing cultured melanoma cells or inhibiting tumor development in animals when administered systemically (16, 24). However, efficacy for preventing cutaneous melanocytic lesion development or for topical applications has not been evaluated.

In this study, the chemopreventive effect of 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 promoting melanoma.
MATERIALS AND METHODS

Reagents. Phenyl butyl isothiocyanate (PBITC) and Phenyl butyl isoselenocyanate (ISC-4) were synthesized by an approach detailed in an earlier report (24, 29).

Cell lines and cell culture. Normal human primary melanocytes FOM 103 (provided by Dr. Herlyn; the Wistar Institute, Philadelphia, PA) were cultured in 1X MCDB 153 (Sigma), 2% FBS, 10% Chelated FBS (Hyclone), 100 nM ET3 (VWR), 10 ng/ml SCF (R&D), 20 pM Cholera Toxin (Sigma), 4.5 ng/ml bFGF (Promega) and 2 mM L-Glutamine (Mediatech) as described previously (30). The human fibroblast FF2441 cell line (received from Dr. Craig Myers lab, Penn State College of Medicine, Hershey, PA) and melanoma cell line UACC 903 (from Mark Nelson at the University of Arizona, Tucson, AZ) were maintained in DMEM (Invitrogen), supplemented with 10% FBS (Hyclone). GFP tagged UACC 903 cells were generated in the Robertson lab (31, 32). WM35 and Sbcl2 (courtesy of Dr. Herlyn; the Wistar Institute, Philadelphia, PA) radial growth phase melanocytic lesion cell lines expressing green fluorescence protein (GFP) were maintained in Tu2% medium as described previously (33). Passage 2 to 5 human foreskin keratinocyte cells (received from Dr. Craig Myers lab, Penn State College of Medicine, Hershey, PA) were cultured in EpiLife E-medium (a serum-free HEPES based medium) containing 1X HKGS consisting of bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human EGF (Cascade Biologics) as detailed previously (34). All of the cell lines used in this manuscript were periodically monitored for phenotype (microscopically examining the cell morphology), by comparing growth properties (doubling time of the cell line using SRB assay) and tumorigenic potential by injecting these cells in to the nude mice to test the tumor forming capacity of these cells. All assessments of cell identity or behavior were similar to that of the original stocks from institutions providing the cell lines.

Determination of cell viability and apoptosis. Viability and IC\textsubscript{50} of melanoma cells following treatment with ISC-4, PBITC or DMSO vehicle was measured using the MTS assay (Promega). Briefly, 5 X 10\textsuperscript{3} melanoma cells or FF2441 or 20 X 10\textsuperscript{3} normal human melanocytes per well in 100 µl DMEM containing 10% FBS were grown in a 96-well plate for 24 or 76 h (~50-70% confluent) and treated with either DMSO vehicle or 2-100 µM of compounds for 24 h and cell viability measured using the MTS assay. IC\textsubscript{50} values for each compound in respective cell lines was determined from three independent experiments using GraphPad Prism version 4.01 (GraphPad software). Apoptosis rates were measured using Apo-ONE Homogenous caspase-3/7 Assay kit (Promega) (16).
**Akt3 knockdown studies.** Animal experimentation was performed according to protocols approved by the IACUC at Pennsylvania State University. Tumor kinetics were measured by subcutaneous injection of 1×10^6 UACC 903 cells nucleofected with control buffer or siAkt3 in 0.2 ml of DMEM supplemented with 10% FBS above both left and right rib cages of 3-4 weeks old female athymic nude-Foxn1nu mice (Harlan Sprague Dawley). Dimensions of developing tumors were measured using calipers on d 10. To measure the apoptotic rates in the tumor xenografts, 5×10^6 UACC 903 cells nucleofected with buffer control or 100 pmoles of siRNA to Akt3 were injected into nude mice as described previously (33). Tumors were harvested 4 days later and fixed in formalin-fixed to assess effect of Akt3 knockdown on apoptosis. Apoptosis rates were measured in formalin-fixed, paraffin-embedded tumor sections using the TUNEL TMR Red Apoptosis kit (Roche) (14, 35). A minimum of 6 different tumors with 4-6 fields per tumor was analyzed and results represented as the average ± SEM.

**Western blot analysis.** For Western blot analysis, floating and attached cells treated with compounds or control vehicle were harvested by addition of lyses buffer and Western blotting undertaken as reported previously (32). Polyvinylidene difluoride membrane (Pall Corporation) blots were probed with antibodies according to each supplier’s recommendations: phosphorylated-Akt (Ser473), Akt3, cleaved PARP, cleaved caspase and Akt from Cell Signaling Technology; phosphorylated-PRAS40 (Thr246) from Invitrogen and Erk2, α-enolase and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology. Immunoblots were developed using the enhanced chemiluminescence detection system (Pierce Biotechnology).

**Creation of laboratory generated skin.** To create skin in a culture dish, human fibroblasts were trypsinized and resuspended in 10% reconstitution buffer, 10% 10X DMEM (Mediatech), 2.4 µl of 10M NaOH, and 80% Type 1 collagen (Beckton Dickinson) at 2.5×10^5 cells/ml on ice (34). Mixture was aliquoted to 12-well plates and incubated at 37 °C for 3 h. 1 ml E-medium was then added to each well to equilibrate the dermis and incubated for 2 d as reported previously (36). After 2 d, keratinocytes and WM35-GFP or UACC 903-GFP cells were trypsinized and resuspended at a ratio of 1:10 or 1:5 respectively in E-media. 1 ml of cell suspension was added on top of each dermal layer. 2 d later reconstructed skin was transferred onto wire grids and fed via diffusion from E-media below the platform. Skin reconstructs were maintained in the E-media composed of Dulbecco’s Modified Eagle Medium (10 mg/ml), Ham F12 powder (2.7 mg/ml), NaHCO₃ (3.1 mg/ml), Adenine...
(0.1%), Insulin (0.1%) Transferin 0.1%, T₃ (0.1%), Hydrocortizone (0.4 mg/ml), and Cholera Enterotoxin (0.25%).

Quantification of melanocytic lesion development in laboratory-generated skin following topical ISC-4 treatment. On the 6 d following skin generation, skins were treated with vehicle control, PBITC, 7.5 µM or 12.5 µM of ISC-4 for WM35 cells or 12.5 µM or 25 µM of ISC-4 for UACC 903 cells. 10 µM stock solutions of PBITC or ISC-4 were prepared in DMSO and subsequently diluted in PBS for working solutions of 7.5 µM, 12.5 µM or 25 µM. 200 ul of each respective solution was applied to skin once daily for 5 consecutive d using a pipette. Prior to treatment, each skin was photomicrographed using a Nikon SMZ1500 fluorescent microscope equipped with a camera. Total average area occupied by GFP-tagged tumor nodules present in 8-10 skin images was used to quantify melanocytic lesion development using IP Labs image analysis software. On d 11, skins were fixed in 4% paraformaldehyde overnight and transferred 24 h later to a 0.5 M EDTA solution at pH 8.0 (Fisher Scientific) in order to preserve the fluorescence signal. Formalin fixed paraffin embedded sections were stained with H&E for cross-sectional examination of cellular and tissue structure. Frozen skin sections were stained with DAPI for fluorescent microscopy to identify GFP-expressing melanocytic lesion cells.

Animals. Female 3-4 weeks old Athymic Nude-Foxn1nu mice were purchased from Harlan Sprague Dawley and injected subcutaneously with 2.5 X 10⁵ cells in 200 µl DMEM-containing 10% FBS at right and left flanks below the rib cages. After 24 h, mice were randomly caged into 3 groups (n=4 mice/group). ISC-4 stock solution was prepared in DMSO initially and then diluted in acetone. 120 µl of acetone vehicle or ISC-4 containing solution was topically applied daily at the injection site. Dimensions of developing tumors and body weight measurements were recorded on alternate days beginning from d 11, and plotted graphically.

To ascertain mechanism underlying tumor inhibition, UACC 903 cells were injected into nude mice and 6-d later mice were treated daily with ISC-4 solution or vehicle. Size and time matched tumors were harvested at days 11 and 13 to assess changes in cell proliferation and apoptosis. Cell proliferation and apoptosis rates in tumor cells were measured in formalin-fixed, paraffin-embedded tumor sections using purified mouse anti-human Ki-67 (PharMingen) and TUNEL staining kit (Roche). A minimum of 6 different tumors with 4-6 fields per tumor was scored. Tumor was also flash frozen in

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
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liquid nitrogen, pulverized, lysed in protein lysis buffer and Western blotting undertaken, measuring levels of pAkt as well as downstream pPRAS40 in tumors by densitometry as described previously (11).

**Statistical analysis.** Statistical analysis was performed using Prism 4.0 GraphPad Software. Student’s $t$ test and One-way or Two-way Analysis Of Variance (ANOVA) was used for groupwise comparisons, followed by the Tukey’s or Bonferroni’s post hoc tests. Results represent at least three independent experiments and are shown as averages ± S.E.M. Results with a $P$ value less than 0.05 (95% CI) were considered significant.
RESULTS

**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) (16, 24). ISC-4 can kill aggressive invasive advanced stage melanoma cells following systemic administration (16), 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 (37, 38), with the latter developing into non-invasive melanocytic lesions, which can progress into invasive melanoma (34). Therefore, effective topical chemopreventive agents would need to kill early non-invasive 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 µM was required to kill 50% of normal human fibroblast compared 7 µM or 5.0 µM for early stage WM35 or Sbcl2 cells lines derived from an early stage melanocytic lesion in the radial growth phase or 9 µM for invasive UACC 903 melanoma cells derived from an invasive cutaneous melanoma (Table 1). Thus, ISC-4 is 2-5 fold more effective at killing melanocytic lesion or melanoma compared to normal cells, indicating potential utility for topically applications at concentrations <19 µM. PBITC served as a control to demonstrate 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 µM of ISC-4 or control PBITC and cell lysates analyzed by Western blotting. ISC-4 decreased pAkt3 levels at lower concentrations than control PBITC with negligible effect on total Akt protein levels (Fig. 1A). Similarly, a dose dependent decrease in pAkt3 levels was also observed in UACC 903 cells (Fig. 1B). Furthermore, ISC-4 decreased levels of downstream pPRAS40 more effectively that control PBTC, 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 compared to PBITC treated cells (Fig. 1A).
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 demonstrate 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 a rise in levels of apoptosis (Fig. 1C). Decreasing Akt3 protein levels reduce tumor development by ~3-fold (Fig. 1D, p<0.01; t-test; left) by increasing cellular apoptosis by 6-7 fold (Fig. 1D, p<0.01; t-test; right). Thus, decreasing Akt3 activity reduces tumor formation by increasing levels of cellular apoptosis.

**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 24 h treatment with ISC-4, PBITC or vehicle control using MTS and Caspase 3/7 assay kits (Figs. 2A & 2B). Both ISC-4 and PBITC decreased viability (Fig. 2A) by increasing apoptosis (Fig. 2B); 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 (Figs. 2A and 2B; left).

**Topical ISC-4 treatment inhibits melanocytic and melanoma lesion development in laboratory-generated skin.** Human skin can be generated in the laboratory containing melanocytic lesions resembling benign for WM35 (Fig. 3A; left) or aggressive for UACC 903 (Fig. 3B; left) tumors seen in patient skin and effects of anticancer agents evaluated on lesion development in this model (34, 39). Both WM35 and UACC 903 cell lines express green fluorescent proteins (GFP) making melanocytic nodule development detectable and quantifiable using fluorescence microscopy (Figs. 3C & 3D; left).

A decrease in lesion development was observed in laboratory generated skin following ISC-4 treatment compared to controls (Fig. 3). 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 compared to
controls (Figs. 3A, 3B & 3C). In contrast to ISC-4 treated skin having little melanocytic lesion development, control skin contained nests of cells for the WM35-GFP cells line (Figs. 3A & 3C) and many invading disseminating cells for the UACC 903-GFP cell line (Figs. 3B & 3D). At the end of ISC-4 treatment, most melanocytic lesion cells for both cell lines were barely detectable (Fig. 3; 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 (34).

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 pictures per skin totaling 30 pictures) according to the treatment schedule shown in Fig. 4A. Regression in area occupied by WM35-GFP and UACC 903-GFP lesions was observed when treated with ISC-4 compared to controls (Fig. 4B). A similar trend was observed for PBITC but ISC-4 was more effective leading to an 80-90% reduction in average area occupied compared to a 50-60% decrease for PBITC at the end of the treatment (Figs. 4C & 4D; 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 7.5-25 µM, supporting topical use of ISC-4 for preventing melanocytic lesion development.

Topical application of ISC-4 prevents melanocytic lesion development in the skin of nude mice with negligible changes in animal body weight. To demonstrate 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 h later, animals treated topically everyday with ISC-4 or acetone vehicle (Fig. 5A). WM35 cells could not be evaluated in mice since these cells do not grow or form detectable lesions in animals (40, 41). Topical ISC-4 treatment led to a 77% decrease in UACC 903 tumor size compared to vehicle control (Fig. 5A; p<0.001). Body weights of mice treated with ISC-4 compared to control showed no significant differences between groups, suggesting negligible toxicity (Fig. 5A; insert). Thus, use of topical ISC-4 inhibited cutaneous melanocytic lesion development without weight loss that would indicate systemic toxicity.

Mechanistically, ISC-4 inhibits melanocytic lesion development in animals by inhibiting Akt3 activity to trigger tumor cell apoptosis. To determine the mechanism causing 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 to vehicle control treated tumors (Fig. 5B).

To show that decreased Akt3 activity led to an increase in tumor cell apoptosis, rates of apoptosis (TUNEL staining) and proliferation (Ki-67 immunohistochemistry) were compared in size and time matched melanoma tumors excised from ISC-4 treated animals and compared to vehicle control. Tumors harvested at day 11 and 13 from mice treated with ISC-4, showed ~3-fold (Fig. 5C; left, p<0.01; t-test) more TUNEL positive cells compared to 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 led 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, histological 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 (data not shown). Thus, ISC-4 caused negligible systemic toxicity and would be effective for preventing melanocytic lesion development.
DISCUSSION

Sunscreen currently provides a way to prevent development of skin cancer caused by sun exposure by blocking UV rays from reaching and damaging skin cells (1). However, despite the use of sunscreen, melanoma incidence and mortality rates continue to rise (1). UV damage accounts for ~30% of melanomas and an additional ~10% are inherited in families; for the remaining ~70%, the cause remains uncertain (1). Therefore, new novel approaches are needed to augment existing strategies for the prevention of this disease (1-4, 7, 42). With more than 1 billion spent on sunscreen every year in the United States, the market for skin cancer prevention is enormous and continues to grow (43). Therefore, addition of agents such as ISC-4 to sunscreens, body lotions or creams could have a profound impact on this existing market for preventing melanoma.

Topical or localized treatments, such as those being proposed for ISC-4, could permit the use of high local concentrations with minimal toxicity and be useful for treating cutaneous lesions not amenable to surgical removal or other currently available approaches (44). Topical treatment could also allow transdermal delivery of high concentrations of the agent to the melanocytic lesion while minimizing toxicity and other unintended off-target effects associated with systemic administration (45). It is therefore clear that effective prevention and treatment options for melanocytic lesions are urgently needed and that a topical ISC-4 formulation might be a component of this solution.

Currently, surgical excision is the mainstay for eliminating early melanocytic lesions or preventing development into more aggressive cancer (9); however, topical ISC-4 treatment could potentially be an adjunct or alternative to surgery for some patients (46). Targeting signaling cascades, such as the Akt3 pathway involved in early melanoma development by decreasing inhibitory MAP kinase pathway activity and deregulating apoptosis would be the goal of targeted chemoprevention; however, normal cells also utilize these same pathways (47). Therefore, the challenge would be to develop a chemopreventive agent that exerts maximal effect on cancer cells with minimal effect on normal ones. ISC-4 fulfills these criteria since it has negligible effects on normal cells in culture, did not cause obvious damage to keratinocytes, fibroblasts, skin architecture or organ function, thereby supporting its potential for topical use.

Naturally occurring chemicals can be useful for preventing cancer. Plants are one source for these agents providing many nutrients, antioxidants, phyto-chemicals and minerals such as selenium
Isothiocyanates from which ISC-4 was developed are naturally occurring phytochemicals found in cruciferous vegetables with many anti-cancer properties. Among the most well studied isothiocyanates are phenethyl isothiocyanate, sulforaphane, indole-3-carbinol (2, 3). Selenium is an essential mineral element present in many dietary sources and its deficiency has been reported to occur in certain cancers including melanoma (25, 26, 28, 49). Dietary selenium supplementation above the recommended daily allowance has been found to lower incidence of some cancers, particularly prostate cancer (50). The exact mechanisms for the anti-cancer effects of selenium are not fully known (14), but may involve antioxidant protection, altered carcinogen metabolism, decreased inflammation, enhanced immune protection, induction of cell cycle arrest and apoptosis, and inhibition of tumor invasion (16, 17). Since both isothiocyanates and selenium possess independent anti-cancer properties, incorporating selenium structurally into PBITC has been shown to enhance efficacy of ISC-4 for inhibiting cancer (11, 19) and for preventing cutaneous melanocytic lesion development in this study.

Prior reports have shown that control compounds structurally similar to ISC-4 had little effect on melanoma cell survival; therefore, the tumor inhibitory effects mediated by ISC-4 are dependent on the structure of the compound and the presence of selenium in this molecule, which confers its enhanced chemopreventive activity (16, 24). Compared with the chemopreventive effects of other selenium-containing agents, ISC-4 performs a unique role in melanoma by targeting Akt3 signaling to promote apoptosis and prevent tumor development with negligible toxicity at biologically effective topical doses. The longer alkyl chain length increases lipophilicity and thereby cellular uptake (51). Selenium in turn enhances the potency for Akt3 inhibition (17, 26). Efficacy for inhibiting cutaneous melanocytic lesion development in this study suggests that ISC-4 can permeate the skin easily reaching the cancer cells where it performs its inhibitory role. Therefore, ISC-4 represents a promising adjunct to currently available chemopreventive agents or for use on cutaneous melanomas for which surgical excision is not an option.

In conclusion, this study demonstrates the utility of topical ISC-4 for preventing 80-90% of cutaneous melanocytic lesion development in preclinical models by targeting the Akt3 signaling cascade. Thus, topical ISC-4 has potential to delay or slow melanocytic lesion or melanoma development in preclinical models and could impact melanoma incidence if similar results are observed in humans.
ACKNOWLEDGEMENTS:

Dr. SubbaRao Madhunapantula, Ph.D. is thanked for providing technical assistance.
FIGURE LEGENDS

Figure 1. Targeting Akt3 using siRNA or ISC-4 induces apoptosis in cultured melanoma cells and melanoma tumor xenografts. A & 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 d 10 compared to control cells (left). Results of three independent experiments were plotted; bars, tumor size mean ± S.E.M. Rates of tumor cell apoptosis measured by TUNEL staining, increased with decreasing Akt3 protein levels (right).

Figure 2. 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 (UACC 903) and normal epidermal human melanocytes were treated with increasing concentrations of PBITC or ISC-4 for 24h. Cell viability was measured by MTS assay. Results of three independent experiments were plotted; bars, mean ± S.E.M. B. ISC-4 treatment triggers apoptosis. PBITC or ISC-4 treatment for 24h increased caspase-3, 7 activity in cell culture media. Bar graph represents fold increase over DMSO treated control. Results of three independent experiments were plotted; bars, mean ± S.E.M.

Figure 3. 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 d 6 to 11 at which point skin was processed for microscopy. For each treatment group 3 skins were used. For each skin, 8-10 pictures were taken daily, and the average areas occupied by the green nodules were quantified using the IPLab software. H&E stained cross-section of the skin reconstructs at d 11.
containing: A. WM35-GFP; or B. UACC 903-GFP (200 X). C. Fluorescence microscopy of frozen DAPI stained cross-sections of the skin containing GFP-tagged WM35-GFP (40X). D. Image is a fluorescent photomicrograph from top of skin surface showing WM35-GFP and UACC 903-GFP untreated or treated with ISC-4 (40X). Images show inhibition of cutaneous melanocytic lesion or invasive melanoma development following ISC-4 treatment.

Figure 4. Melanocytic lesion or melanoma development following ISC-4 treatment in laboratory-generated skin. A. ISC-4 treatment regime. Laboratory generated skin at d 6 was treated daily with ISC-4, PBITC or vehicle up to d 10. B. Area occupied by GFP-tagged melanocytic lesions following ISC-4 treatment. Area occupied by fluorescent melanocytic or melanoma cells in laboratory-generated skin was quantified daily using fluorescence microscopy and 2-dimensional image analysis software; values, mean ± S.E. (3 skins/group were used and for each skin 10 images were photographed). Daily treatment led to an 80-90% decrease in size by d 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 d 6 with 7.5-25 μM of ISC-4 or PBITC daily for 5 consecutive d and lesion development quantified and compared at d 11; values, mean ± S.E. ISC-4 decreased lesion development 50-70% (p< 0.001; One-Way ANOVA) more effectively than PBITC when compared to controls.

Figure 5. 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 4-6 weeks old female athymic nude mice and 24 h later ISC-4 or acetone vehicle applied topically each day. Tumors were measured on alternate d until d 29; values, mean ± S.E. Topical application of ISC-4 led to a 77% decrease (p<0.001) in tumor volume beginning from days 19 compared to 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 d 13 for Western blot analysis; values, mean ± S.E. ISC-4 decreased pAkt (p<0.05; t-test) and downstream pPRAS40 (p<0.001; t-test) levels by 60-70% in ISC-4 treated tumor lysates compared to controls. C. ISC-4 increased tumor cell apoptosis but has a non-significant 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 ± S.E. ISC-4 increased melanoma tumor cells apoptosis at days 11 and 13 compared to vehicle control (P< 0.01; One-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 ± S.E.
REFERENCES

48. Finley JW, Davis CD. Selenium (Se) from high-selenium broccoli is utilized differently than selenite, selenate and selenomethionine, but is more effective in inhibiting colon carcinogenesis. Biofactors 2001; 14: 191-6.
Table 1: IC$_{50}$ of ISC-4 on normal, melanocytic lesion and melanoma cell lines

<table>
<thead>
<tr>
<th></th>
<th>PBTC (Phenylbutyl isothiocyanate)</th>
<th>ISC-4 (Phenylbutyl isoselenocyanate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH$_2$CH$_2$CH$_2$N=C=S</td>
<td>CH$_2$CH$_2$CH$_2$N=C=Se</td>
</tr>
<tr>
<td><strong>Normal cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Fibroblast</td>
<td>37±5</td>
<td>24±3</td>
</tr>
<tr>
<td>Human Melanocytes</td>
<td>30±5</td>
<td>19±3</td>
</tr>
<tr>
<td><strong>Melanoma cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WM35</td>
<td>9±0.4</td>
<td>7±2</td>
</tr>
<tr>
<td>Sbcl2</td>
<td>10±2</td>
<td>5±0.4</td>
</tr>
<tr>
<td>UACC 903</td>
<td>17±2</td>
<td>9±0.9</td>
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</tbody>
</table>
A. WM35 (Melanocytic Lesion Cells)

B. UACC 903 (Invasive Melanoma Cells)

C. UACC 903 (Invasive Melanoma Cells)

D. UACC 903 (Invasive Melanoma Cells)
**A  Cell Viability (MTS Assay)**

- **Melanocytes (Normal Cells)**
  - Concentrations (μM): 2.5, 5, 15, 35
  - % of DMSO control

- **WM35 (Melanocytic Lesion Cells)**
  - Concentrations (μM): 1.6, 3.1, 4.5, 6.3, 12.5
  - % of DMSO control

- **UACC 903 (Invasive Melanoma Cells)**
  - Concentrations (μM): 3.12, 6.25, 12.5, 25, 50
  - % of DMSO control

**B  Apoptosis (Caspase - 3/7 Activity)**

- **Melanocytes (Normal Cells)**
  - Concentrations (μM): 2.5, 5, 15, 35
  - Fold increase over DMSO control

- **WM35 (Melanocytic Lesion Cells)**
  - Concentrations (μM): 1.6, 3.1, 4.5, 6.3, 7.5
  - Fold increase over DMSO control

- **UACC 903 (Invasive Melanoma Cells)**
  - Concentrations (μM): 3.13, 6.25, 12.5, 25
  - Fold increase over DMSO control
H&E Stained cross section of skin reconstruct

A

WM05-GFP (untreated)  
Epidemis  
Dermis  
200X

WM05-GFP (ISC-4 treated)  
Epidemis  
Dermis  
200X

UACC 903-GFP (untreated)  
200X

B

UACC 903-GFP (ISC-4 treated)  
200X

Cross section of skin reconstruct

C

WM05-GFP (untreated)  
40X

WM05-GFP (ISC-4 treated)  
40X

Fluorescent photomicrograph from top of skin surface

D

WM05-GFP (untreated)  
4.6X  
UACC 903-GFP (untreated)  
4.6X

WM05-GFP (ISC-4 treated)  
4.6X  
UACC 903-GFP (ISC-4 treated)  
4.6X
Melanoma Chemoprevention in Skin Reconstructs and Mouse Xenografts using Isoselenocyanate-4

Natalie Nguyen, Arati Sharma, Nhung Nguyen, et al.


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