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

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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 lead 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. Cancer Prev Res; 4(2); 248–58. ©2010 AACR.

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). 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 (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.

Mechanistically, ISC-4 prevented melanoma by decreasing Akt3 signaling that lead 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. Cancer Prev Res; 4(2); 248–58. ©2010 AACR.
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 anticancer properties (17–19), providing protection 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 phosphoinositide 3-kinase pathway (22, 23). Unfortunately, isothiocyanates had low chemotherapeutic potency on melanoma cells requiring high concentrations for therapeutic efficacy, which made these compounds unsuitable for therapeutic or preventive agents (16, 24). 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 (16, 24). Selenium was incorporated into the structure, as it can be an effective chemopreventive agent (25, 26) and its deficiency occurs frequently in cancer patients including those with a diagnosis of metastatic melanoma (27). 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 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.

Materials and Methods

Reagents

Phenyl butyl isothiocyanate (PBITC) and phenyl butyl isoselenocyanate were synthesized by an approach detailed in an earlier report (24, 28).

Cell lines and cell culture

Normal human primary melanocytes FOM 103 (provided by Dr. Herlyn, the Wistar Institute, Philadelphia, PA) were cultured in 1 x MCDB 153 (Sigma), 2% FBS, 10% chelated FBS (Hyclone), 100 nmol/LET3 (VWR), 10 ng/mL SCF (R&D Systems), 20 pmol/L cholera toxin (Sigma), 4.5 ng/mL bFGF (Promega), and 2 mmol/L-glutamine (Mediatech) as described previously (29). The human fibroblast cell line FF2441 (received from Dr. Herlyn, the Wistar Institute, Philadelphia, 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). Green fluorescent protein (GFP)-tagged UACC 903 cells were generated in the Robertson laboratory (30, 31). WM35 and Sbcl2 (courtesy of Dr. Herlyn, the Wistar Institute, Philadelphia, PA), radial growth phase melanocytic lesion cell lines expressing GFP, were maintained in Tu2% medium as described previously (32). Passage 2 to 5 human foreskin keratinocyte cells (received from Dr. Craig Myers’ laboratory, Penn State College of Medicine, Hershey, PA) were cultured in Epilife E-medium (a serum-free HEPES-based medium) containing 1 x HKGS, consisting of bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human EGF (Cascade Biologics) as detailed previously (33). All of the cell lines used in this article were periodically monitored for phenotype (microscopically examining the cell morphology), by comparing growth properties (doubling time of the cell line by SRB assay) and tumorigenic potential by injecting these cells into nude mice to test the tumor-forming capacity of these cells. All assessments of cell identity or behavior were similar to those of the original stocks from institutions providing the cell lines.

Determination of cell viability and apoptosis

Viability and IC_{50} of melanoma cells following treatment with ISC-4, PBITC, or DMSO vehicle were measured using the MTS assay (Promega). Briefly, 5 x 10^3 melanoma cells or FF2441 or 20 x 10^3 normal human melanocytes per well in 100 mL DMEM containing 10% FBS were grown in a 96-well plate for 24 or 76 hours (~50%-70% confluent) and treated with either DMSO vehicle or 2 to 100 μmol/L of compounds for 24 hours and cell viability was measured using the MTS assay. IC_{50} values for each compound in respective cell lines were determined from 3 independent experiments, using GraphPad Prism version 4.01 (GraphPad Software). Apoptosis rates were measured using Apo-ONE Homogenous Caspase-3/7 Assay kit (Promega; ref. 16).

Akt3 knockdown studies

Animal experimentation was carried out according to protocols approved by the IACUC (Institutional Animal Care and Use Committee) at Pennsylvania State University. Tumor kinetics were measured by subcutaneous injection of 1 x 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 to 4 weeks old female athymic Nude-Foxn1nu mice (Harlan Sprague Dawley). Dimensions of developing tumors were measured using calipers on day 10. To measure the apoptotic rates in the tumor xenografts, 5 x 10^6 UACC 903 cells nucleofected with buffer control or 100 pmols of siRNA to Akt3 was injected into nude mice as described previously (32). Tumors were harvested 4 days later and fixed in formalin to assess the effect of Akt3 knockdown on apoptosis. Apoptosis rates were measured in formalin-fixed, paraffin-embedded tumor sections with the TUNEL TMR
Red Apoptosis kit (Roche; ref. 14, 34). A minimum of 6 different tumors, with 4 to 6 fields per tumor, were analyzed and results were 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 the addition of lysis buffer and Western blotting was undertaken as reported previously (31). 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-elonase and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology.

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% 10× DMEM (Mediatech), 2.4 μL of 10 mol/L NaOH, and 80% type I collagen (Beckton Dickinson) at 2.5 × 10^6 cells per mL on ice (33). Mixture was aliquoted to 12-well plates and incubated at 37°C for 3 hours. One milliliter of E-medium was then added to each well to equilibrate the demin and incubated for 2 days as reported previously (35). After 2 days, 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-medium. One milliliter of cell suspension was added on top of each dermal layer. Two days later, reconstructed skin was transferred onto wire grids and fed via diffusion from E-medium below the plate. Skin reconstructs were maintained in the E-media grids and fed via diffusion from E-media below the plate. On day 6 following skin generation, skins were treated with vehicle control, PBITC, 7.5 or 12.5 μmol/L of ISC-4 for UACC 903 cells were injected into nude mice and 6 days 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 (PharMin- gen) and TUNEL staining kit (Roche). A minimum of 6 different tumors with 4 to 6 fields per tumor was scored. Tumor was also flash-frozen in 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).

**Quantification of melanocytic lesion development in laboratory-generated skin following topical ISC-4 treatment**

On day 6 following skin generation, skins were treated with vehicle control, PBITC, 7.5 or 12.5 μmol/L of ISC-4 for WM35 cells, or 12.5 or 25 μmol/L of ISC-4 for UACC 903 cells. Stock solutions (10 μmol/L) of PBITC or ISC-4 were prepared in DMSO and subsequently diluted in PBS for working solutions of 7.5, 12.5, or 25 μmol/L. Two hundred microliters of each respective solution was applied to skin once daily for 5 consecutive days with a pipette. Prior to treatment, each skin was photomicrographed using a Nikon SMZ1500 fluorescence microscope equipped with a camera. Total average area occupied by GFP-tagged tumor nodules present in 8 to 10 skin images was used to quantify melanocytic lesion development using IP Labs image analysis software. On day 11, skins were fixed in 4% paraformaldehyde overnight and transferred 24 hours later to a 0.5 mol/L EDTA solution at pH 8.0 (Fisher Scientific) in order to preserve the fluorescence signal. Formalin-fixed, paraffin-embedded sections were stained with hematoxyline and eosin (H&E) for cross-sectional examination of cellular and tissue structures. Frozen skin sections were stained with 4′, 6-diamidino-2-phenylindole (DAPI) for fluorescent microscopy to identify GFP-expressing melanocytic lesion cells.

**Animals**

Female 3 to 4 weeks old athymic Nude-Foxn1nu mice were purchased from Harlan Sprague Dawley and injected subcutaneously with 2.5 × 10^5 cells in 200 μL DMEM-containing 10% FBS at right and left flanks below the rib cages. After 24 hours, mice were randomly caged into 3 groups (n = 4 mice per group). ISC-4 stock solution was prepared in DMSO initially and then diluted in acetone. One hundred twenty microliters 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 day 11, and plotted graphically. To ascertain mechanism underlying tumor inhibition, UACC 903 cells were injected into nude mice and 6 days 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 (PharMin- gen) and TUNEL staining kit (Roche). A minimum of 6 different tumors with 4 to 6 fields per tumor was scored. Tumor was also flash-frozen in 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 carried out using Prism 4.0 GraphPad Software. Student’s t test and 1-way or 2-way ANOVA was used for groupwise comparisons, followed by Tukey’s or Bonferroni’s post hoc tests. Results represent at least 3 independent experiments and are shown as averages ± SEM. Results with a value of P < 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 thiocyanates by increasing the alkyl carbon chain length to contain 4 carbons and replacing sulfur with selenium (structure shown in Table 1; refs. 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 (36, 37), with the latter developing into noninvasive melanocytic lesions, which can progress into invasive melanoma (33). 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 903 melanoma 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 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 903 melanoma 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. 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 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. 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 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. 1C). Decreasing Akt3 protein levels reduce tumor development by approximately 3-fold (Fig. 1D, *P* < 0.01; *t* test; left) by increasing cellular apoptosis by 6- to 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 a 24-hour treatment protocol with ISC-4, PBITC, or vehicle control with MTS and Caspase 3/7 Assay kits (Fig. 2A and B). 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 (Fig. 2A 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. 3A; left) or aggressive for UACC 903 (Fig. 3B; 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 WM35 and UACC 903 cell lines express GFPs, making melanocytic nodule development detectable and quantifiable by fluorescence microscopy (Fig. 3C and D; left).

A decrease in lesion development was observed in laboratory-generated skin following ISC-4 treatment compared with controls (Fig. 3). H&E-stained and fluorescent images of cross-sections of skin at day 11 showed dramatically fewer melanocytic lesion or melanoma cells in the skin than controls (Fig. 3A–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. 3A and C) and many invading disseminating cells for the UACC 903-GFP cell line (Fig. 3B and D). 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 (33).

To assess the effect of ISC-4 treatment on melanocytic lesion development over time, 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 4A. Regression in area occupied by WM35-GFP and UACC 903-GFP lesions was observed when treated with ISC-4 compared with controls (Fig. 3A). 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 \( \mu \text{mol/L} \), 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 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. 5A). WM35 cells could not be evaluated in mice, as these cells do not grow or form detectable lesions in animals (39, 40). Topical ISC-4 treatment led to a 77% decrease in UACC 903 tumor size compared with vehicle control (Fig. 5A; P < 0.001). Body weights of mice treated with ISC-4 compared with control showed no significant differences between groups, suggesting negligible toxicity (Fig. 5A; insert). Thus, the 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 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 (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 increase (1). UV damage accounts for approximately 30% of melanomas and an additional approximately 10% are inherited in families; for the remaining approximately 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, 41). 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 (42). 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 (43). 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 (44). 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 (45). 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 (46). 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, as it has negligible effects on normal cells in culture and does not cause obvious damage to keratinocytes, fibroblasts, skin architecture, or organ function, thereby supporting its potential for topical use.

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 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 range of serum levels for nude mice. No significant differences were observed, indicating negligible vital organ related toxicity; values, mean ± SE.
from which ISC-4 was developed are naturally occurring phytochemicals found in cruciferous vegetables with many anticancer properties. Among the most well-studied isothiocyanates are phenethyl isothiocyanate, sulforaphane, and 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, 27, 48). Dietary selenium supplementation above the recommended daily allowance has been found to lower incidence of some cancers, particularly prostate cancer (49). The exact mechanisms for the anticancer 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). Because both isothiocyanates and selenium possess independent anticancer properties, incorporating selenium structurally into PBITC has been shown to enhance efficacy of ISC-4 both 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 plays 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 (50). Selenium, in turn, enhances the potency for Akt3 inhibition (17). 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 plays 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 shows the utility of topical ISC-4 for preventing 80% to 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

Dr. Subha Rao Madhunapantula is thanked for providing technical assistance.

Grant Support

This work was supported by The American Cancer Society (RSG-04-053-01-MC) and the American Cancer Society (to G.P. Robertson); NIH contract N02-CP-56603 (to S. Amin); Elsa U. Pardee Foundation (to A. Sharma and A.K. Sharma); and Melanoma Research Foundation with support in part from the Mike Geltrude Foundation and PA Department of Health (to A. Sharma). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 6, 2010; revised May 6, 2010; accepted October 29, 2010; published OnlineFirst November 19, 2010.

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