Research Article

β-Escin Inhibits NNK-Induced Lung Adenocarcinoma and ALDH1A1 and RhoA/Rock Expression in A/J Mice and Growth of H460 Human Lung Cancer Cells

Jagan M.R. Patlolla, Li Qian, Laura Biddick, Yuting Zhang, Dhimant Desai, Shantu Amin, Stan Lightfoot, and Chinthalapally V. Rao

Abstract

Lung cancer is the leading cause of cancer-related deaths. β-Escin, a triterpene saponin isolated from horse chestnut seeds, was tested for inhibition of lung adenoma and adenocarcinoma induced by the tobacco carcinogen 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice; and its possible mode of action was evaluated using the H460 human lung cancer cell line. At 6 weeks of age, 35 mice were fed AIN-76A–modified diet, and one week later, lung tumors were induced with a single intraperitoneal (i.p.) injection of 10 μmol NNK/mouse. Three weeks after the NNK treatment, groups of mice were fed either control or experimental diets containing 500 ppm for 20 weeks (10 control, 5 β-escin) or 36 weeks (15 control, 5 β-escin) and evaluated for lung tumor via histopathologic methods. Administration of 500 ppm β-escin significantly suppressed lung tumor (adenoma + adenocarcinoma) formation by more than 40% (P < 0.0015) at 20 weeks and by 53.3% (P < 0.0001) at 37 weeks. β-Escin inhibited NNK-induced lung adenocarcinoma formation by 65% (P < 0.001) at 20 weeks and by 53% (P < 0.0001) at 37 weeks. Immunohistochemical analysis revealed that lung tumors from mice exposed to β-escin showed significantly reduced aldehyde dehydrogenase (ALDH)1A1 and phospho-Akt (p-Akt) expression when compared with those in mice fed control diet. Aldefluor assay for ALDH revealed that among H460 lung cancer cells treated with different concentrations of β-escin (0–40 μmol/L), the subpopulation of cells with elevated ALDH activity was inhibited significantly. Our findings suggest that β-escin inhibits tobacco carcinogen-induced lung tumor formation by modulating ALDH1A1-positive cells and RhoA/Rock signaling. Cancer Prev Res; 6(10); 1140–9. ©2013 AACR.

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Introduction

Lung cancer is the most common cause of cancer mortality in both men and women in the United States and it is a major public health problem worldwide (1). Despite significant advances in chemotherapeutic, radiotherapeutic, and targeted treatment approaches for lung cancer, the long-term survival of patients with advanced-stage lung cancer remains low (2). To date, human prevention trials in the area of lung cancer have shown minimal promise (3). Thus, there is a need for identifying new chemopreventive agents with novel mechanisms of action for prevention of lung cancer. Here, we tested the chemopreventive properties of β-escin, or aescin, a triterpene saponin compound from extracts of horse chestnut (Aesculus hippocastanum) seeds (structure shown in Fig. 1A). β-Escin has been used widely to treat chronic venous insufficiency (4), hemorrhoids, and postoperative edema resulting from surgery (5). Studies from our laboratory showed that β-escin inhibits the growth of colon cancer cells and inhibits chemically induced colon carcinogenesis in rats (6). In recent years, several studies have shown that β-escin possesses anticancer activity by inhibiting growth and inducing apoptosis in various human cancer cell lines derived from lung (7), pancreatic (8), and liver (9) cancers and in leukemia and multiple myeloma (10).

Recent studies have attributed resistance of tumors to various therapies and recurrence of tumor growth to the presence of cancer stem cells (CSC; refs. 11, 12). Evidence suggests that human lung cancers harbor CSC populations (13, 14). Targeting and specifically eradicating these populations of cells with nontoxic chemopreventive agents could provide an ideal approach to preventing the cancers. Several investigators isolated and characterized CSCs using a variety of stem cell markers including CD133 (15) and aldehyde dehydrogenases

Authors’ Affiliations: 1Center for Cancer Prevention and Drug Development, Department of Medicine, Hem-Onc Section, PCS Oklahoma Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and 2Department of Pharmacology, College of Medicine, Penn State Hershey Medical Center, Hershey, Pennsylvania

Corresponding Authors: Chinthalapally V. Rao, Center for Cancer Prevention and Drug Development, 975 NE 10th Street, BRC 1203, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104. Phone: 405-271-3224; Fax: 405-271-3225; E-mail: cv-rao@ouhsc.edu; and Jagan M.R. Patlolla, E-mail: Jagan-Patlolla@ouhsc.edu

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(ALDH; refs. 16). ALDHs are a family of intracellular enzymes that participate in cellular detoxification and differentiation and contribute to drug resistance (17). Previous studies have reported that 2 ALDH isozymes, ALDH1A1 and ALDH3A1, are overexpressed in tumors compared with normal lung (16, 18) and in lung cancer cell lines (18, 19). ALDH1A1 is associated with chemoresistance and targeting ALDH1A1 sensitizes resistant tumor cells to chemoprevention.

We have evaluated the chemopreventive efficacy of β-escin on NNK-induced lung adenoma and adenocarcinoma formation in female A/J mice. We investigated the effectiveness of β-escin on progression from adenoma to adenocarcinoma by administering the agent either short-term or long-term, and we tested whether β-escin influences ALDH1A1+ stem cells and markers of lung tumor progression.

Materials and Methods

Materials

β-Escin (Fig. 1A), more than 98% pure compound, was purchased from Sigma. NNK was provided by the laboratory of Shantu Amin (Department of Pharmacology, Penn State University, Hershey, PA). Anti-RhoA, anti-Rock, anti-p21, anti-phosphorylated Akt (pAkt), and anti-β-actin were purchased from Santa Cruz Biotechnology. Anti-ALDH1A1 antibody (ab52492) was purchased from Abcam. Aldefluor kit was purchased from Stem Cell Technologies.

Cell lines

The human NCI-H460 lung cancer cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Life Technologies Inc.) with 10% FBS under 5% CO₂ at 37°C. The cell line was authenticated by DNA short-tandem repeat analysis by ATCC. H460 cell line was initially expanded and cryopreserved within 1 month of receipt. Cells were typically used for 3 months, and at that time a fresh vial of cryopreserved cells was used. All experiments were carried out with cells grown to 70% to 80% confluence. A stock solution of β-Escin was prepared by solubilizing 10 mg of β-Escin in 5 mL of PBS. To assess growth inhibition and molecular markers, we applied subtoxic concentrations of β-Escin, ranging from 0 to 40 μmol/L.

Animals and experimental diets ± chemopreventive agent. All animal experiments were done in accordance with NIH guidelines and under the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC)-approved protocol (IACUC # 09-067B). Female A/J mice were obtained at age 6 weeks from Jackson Laboratories. Ingredients for the semipurified diets were purchased from Bioserv and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition 76A (AIN-76A) diet containing 20% casein, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mix, 1.2% AIN revised vitamin mix, 0.3% L,L-methionine, and 0.2% choline bitartrate. β-Escin was premixed with a small quantity of casein and

Figure 1. β-Escin structure and bioassay protocol. A, structure of β-escin. B, experimental bioassay protocol to assess the chemopreventive effects of β-escin in NNK-induced lung tumorigenesis in the A/J mouse model. Eight-week-old mice were injected intraperitoneally with NNK (10 μmol/mouse) and maintained on AIN-76A diet for 3 weeks. Three weeks after NNK injection, mice were fed control diet (AIN-76A) or diet containing 500 ppm of β-escin and continued till the end of the study. After 17 or 34 weeks on experimental diets, mice were necropsied and lungs were harvested for evaluation of lung tumors. Detailed information is given in Materials and Methods.
then blended into bulk diet using a Hobart Mixer. Both control and experimental diets were prepared weekly and stored in a cold room. The content of agent in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to ensure uniform distribution.

**Bioassay for tumorigenesis.** The experimental design is summarized in Fig. 1B. Female A/J mice at 6 weeks of age were purchased from the Jackson Laboratory and were maintained in the pathogen-free Rodent Barrier Animal Facility at the University of Oklahoma Health Sciences Center and fed control AIN-76A-modified diet. At 7 weeks of age, mice intended for carcinogen treatment received one dose of 10 μmol NNK by intraperitoneal (i.p.) injection. All mice were weighed once every 2 weeks until termination of the study. Three weeks after NNK treatment, groups of mice (25 mice/group) were fed either control AIN-76A or experimental diet containing 500 ppm β-escin (10 mice/group). Mice were euthanized by CO₂ asphyxiation after 17 weeks (10 mice from control group and 5 mice from experimental group) or 34 weeks (15 mice from control group and 5 mice from experimental group) of exposure to test agent in diet. At necropsy, lungs were lavaged, perfused, and fixed in phosphate-buffered formalin, transferred within 2 days to 70% alcohol, and evaluated under a dissecting microscope for number of tumors and tumor size. Before the fixation, tumors on the lung surface were enumerated by at least 2 experienced readers, blinded to sample identifiers, using a dissecting microscope. Tumor diameters were measured using Fisher brand digital calipers.

**Tumor histology**

Fixed lung samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). H&E-stained lung sections from 3 to 5 separate sections cut at predetermined depths were evaluated by a board-certified pathologist (S. Lightfoot) for number of adenomas and adenocarcinomas. Tumors were categorized according to criteria of the Mouse Models of Human Cancers Consortium (20). Adenomas were generally less than 5 mm in diameter well circumscribed, with areas of proliferative cuboidal to columnar cells lining an alveolus. Adenocarcinomas were typically larger than 5 mm in diameter and showed invasion and loss in alveolar architecture, increased nuclear/cytoplasmic ratio, cellular atypia, a large mass of undifferentiated cells, and nuclear pleomorphism.

**Histology and immunohistochemistry**

The effects of β-escin on expression of proliferating cell nuclear antigen (PCNA), p21, and ALDH1A1 were evaluated by immunohistochemistry. Briefly, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in PBS. Antigen retrieval was carried out by treating sections in 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 5 minutes. Nonspecific binding sites were blocked by incubation with 2% bovine serum albumin (BSA); then, sections were incubated overnight at 4°C with 1:50 dilutions of antibodies against PCNA, p21, and ALDH1A1. After several washes with PBS, tissue sections were incubated with appropriate secondary antibodies for 2 hours before incubation with avidin–biotin complex reagent (Invitrogen) and then with the chromogen 3-diaminobenzidine (DAB) for 3 minutes. Slides were rinsed and counterstained with hematoxylin. Nonimmune rabbit immunoglobulins were substituted for primary antibodies as negative controls. Staining for positive expression was observed under an Olympus microscope 1 × 701 and digital computer images were recorded with an Olympus DP70 camera.

**Aldefluor assay and flow cytometry**

The Aldefluor Kit (Aldagen Inc.) was used to profile and separate H460 cells with high and low ALDH activity (19, 21). Briefly, H460 cells were treated with different concentrations of β-escin from 0 to 40 μmol/L and then were incubated in Aldefluor assay buffer containing the ALDH protein substrate BODIPY-aminoacetalddehyde (BAAA) for 45 minutes at 37°C. Cells that could catalyze conversion of BAAA to its fluorescent product BODIPY-aminoacetate (BAA) were considered ALDH⁺. Samples treated with the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB) during the incubation served as negative controls. Sorting gates for fluorescence-activated cell sorting (FACS) were drawn relative to cell baseline fluorescence as determined by the addition of DEAB. Nontiable cells were identified by propidium iodide inclusion. Cells were sorted by an influx cell sorter (Cytopeia) or BD Aria (BD Biosciences). FACS analyses were conducted on a FACScan or FACSCalibur flow cytometer (BD Bioscience), and figures were produced using Spigot 6.3 software (5).

**Western blot analysis of protein expression**

Lung tumors harvested from mice from control and test agent treatment groups were homogenized and lysed in ice-cold lysis buffer (Sigma). After a brief vortexing, the lysates were separated by centrifugation at 12,000 × g for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad Protein Assay Reagent. An aliquot (50 μg protein/lane) of the total protein was separated with 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% milk powder, membranes were probed for expression of p21, Aldh1A1, Rho A, Rock, and β-actin in hybridizing solution [1:500 in Tris-buffered saline (TBS)-Tween 20] using the respective primary antibodies and then incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Detection was conducted using the SuperSignal West Pico Chemiluminescence procedure (Pierce). The bands were captured on Ewen Parker Blue sensitive X-ray films and quantified by densitometry. We reprobed every blot for β-actin but we present here only one β-actin blot from each of the two intervals of test agent exposure: 17 and 34 weeks. Band intensity was calculated by densitometry using the ImageJ software provided by NIH as an open free software. Results were expressed as densitometry units (DU).
RNA extraction and conventional PCR for p21 expression in Aldh1A1− and Aldh1A1+ cells

Total RNA was isolated from control and 20 μmol/L β-escin–treated ALDH+ and ALDH− cells using TRIzol Reagent (Invitrogen) and subjected to conventional PCR using an iScript cDNA synthesis kit (Bio-Rad). Oligonucleotide primer sequences used for p21 were as follows: 5′-GCTGAGGTTCCCGACGT-3′, sense; 5′-GAAATCTGTGATGTCGTCG-3′, antisense; conditions were as follows: denaturation at 94°C for 3 minutes was followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. PCR was done using the Taq polymerase, 10 mmol/L deoxynucleoside triphosphates, and buffers from Invitrogen. The PCR products were electrophoresed on 2% agarose gels with ethidium bromide staining. A representative H&E staining of lung tumors from mice administered β-escin for 17 weeks showed a 31.8% (P<0.0001) reduction in lung adenomas; lung adenocarcinomas were significantly suppressed by 65.5% (P<0.0001). A similar trend was observed in the mice exposed to β-escin for 34 weeks; NNK-induced adenomas and adenocarcinomas were inhibited by 38.5% (P<0.05) and 56% (P<0.0001), respectively (Fig. 2E and F). Thus, we observed a limited effect on lung adenoma formation; but there was a significant reduction in tumor progression from adenoma to adenocarcinoma in the β-escin–treated mice.

Statistical analysis

Differences in body weights among groups were analyzed by ANOVA. Adenoma and adenocarcinoma multiplicities (number of tumors/mouse) are expressed as means ± SD. Tumor incidence was analyzed by Fisher exact test, and tumor multiplicity was analyzed by Student t test. Protein expression and proliferative indices are expressed as means ± SEM and were analyzed by unpaired, one-tailed t test with Welch correction. Differences were considered statistically significant at P<0.05.

Results

β-Escin did not exhibit toxicity in female A/J mice

β-Escin dose selection for efficacy studies was based on our previous toxicity studies in rodents (6) and an approximate dose used clinically in humans (22). Administration of 500 ppm β-escin for either 17 or 34 weeks did not cause any body weight loss or any other histologic toxicity in major organ sites (data not shown). Chronic exposure of β-escin failed to show any hemolysis in animals in our studies. Overall, chronic dietary administration of β-escin lacks overt toxicities in female A/J mice.

Dietary administration of β-escin significantly inhibited lung tumor formation and delayed the progression of adenoma to adenocarcinoma formation

The effect of β-escin on total lung tumor formation was analyzed in Fig. 2A–F. Mice fed control diet exhibited 100% tumor incidence at 20 and 37 weeks after treatment with the carcinogen NNK. Control diet mice killed at 20 weeks after NNK treatment showed an average of 9.4 ± 2.3 lung adenomas and 5.8 ± 1.8 lung adenocarcinomas (means ± SD); that is, 61.8% of the tumors were adenomas and 38.5% were adenocarcinomas (Fig. 2C and D). Mice fed control diet and killed at 37 weeks after NNK treatment showed 2.2 ± 0.8 adenomas and 16.2 ± 2.8 adenocarcinomas (i.e., 11.95% of the tumors were adenomas and 88.4% were adenocarcinomas; Fig. 2E and F), reflecting an increased progression of adenomas to adenocarcinomas, as anticipated. Mice administered 500 ppm of β-escin for 17 and 34 weeks showed an inhibition of 39% and 53%, respectively, in NNK-induced lung tumor formation (Fig. 2A and B). The effects of β-escin on lung adenoma and adenocarcinoma multiplicities are summarized in Fig. 2C and D. Mice administered β-escin for 17 weeks showed a 23% (P<0.035) reduction in lung adenomas; lung adenocarcinomas were significantly suppressed by 65.5% (P<0.0001). A similar trend was observed in the mice exposed to β-escin for 34 weeks; NNK-induced adenomas and adenocarcinomas were inhibited by 38.5% (P<0.05) and 56% (P<0.0001), respectively (Fig. 2E and F). Thus, we observed a limited effect on lung adenoma formation; but there was a significant reduction in tumor progression from adenoma to adenocarcinoma in the β-escin–treated mice.

Modulation of PCNA, p21, and ALDH1A1 expression by β-escin in NNK-induced lung adenocarcinomas

NNK-induced lung tumor both adenoma and adenocarcinomas were histopathologically evaluated after the H&E staining. A representative H&E staining of lung tumors from mice fed either control diet or β-escin has been shown in Fig. 3A. On the basis of the histopathology, NNK-induced lung tumors either adenomas or adenocarcinomas are discussed. Adenocarcinomas in the control group were with varying degrees of differentiation and were characterized by complete loss of normal alveolar architecture when compared to β-escin treatment group (Fig. 3A). Our observation suggest that β-escin delays the adenoma to adenocarcinoma progression and the major histologic difference we observed in the β-escin–treated group is a moderate decrease in the amount of cytoplasm and small degree of pleomorphism, without losing the alveolar architecture, thus retaining the adenoma structure instead of adenocarcinoma (Fig. 3A) at 34-week stage in the treated group. β-Escin effects on proliferation were assessed by PCNA labeling of cells (Fig. 3B). The PCNA labeling index was significantly lowered in
tumors of the mice treated with β-escin than in tumors of mice fed control diet. Quantification of PCNA staining showed a labeling index of 68 ± 4.8 (mean ± SEM) in adenocarcinomas from mice fed the control diet as compared with 35 ± 2.6 (mean ± SEM) in animals fed β-escin, reflecting a decrease in the proliferation index by 48% (P < 0.0001; Fig. 3B). As shown in Fig. 3C, expression of p21 was reduced significantly in NNK-induced lung adenocarcinomas of mice fed control diet; whereas dietary administration of β-escin resulted in a significant upregulation of p21 protein expression levels in lung adenocarcinomas. Figure 3D summarizes the expression of ALDH1A1 in lung adenocarcinoma from mice fed control diet as well as that from animals fed β-escin. Increased expression of ALDH1A1 is seen around bronchoalveolar regions in NNK-induced lung tumors but not in animals fed β-escin. The density of ALDH1A1 positively stained cells is significantly suppressed in lung tumors of mice fed β-escin as compared with those in mice fed control diet.

Western blot analysis of p21<sup>WAF1/CIP1</sup>, RhoA, Rock, p-Akt, and ALDH1A1 modulation by β-escin

Expression of p21<sup>WAF1/CIP1</sup>, RhoA, Rock, p-Akt, and ALDH1A1 was analyzed further by Western blotting. As shown in Fig. 4A, β-escin caused significant induction of p21<sup>WAF1/CIP1</sup> expression in lung (Fig. 4B). We observed greater decreases in ALDH1A1, RhoA, and Rock proteins in tumors harvested from mice fed β-escin after 34 weeks of exposure compared with mice fed control diet. The densitometry analysis showed a significant increase in p21<sup>WAF1/CIP1</sup> expression levels in β-escin–treated groups compared to the control group (P < 0.02; Fig. 4B). We observed a significant decrease in ALDH1A1, RhoA, and Rock proteins expression (P < 0.02, P < 0.01, and P < 0.03, respectively) in mice fed with β-escin compared with mice fed with control diet (Fig. 4B). The phosphorylation of Akt was inhibited by β-escin; however, total Akt protein levels were unaltered (Fig. 4A).

Modulation of ALDH1A1 mRNA expression in lung tumors and induction of p21 in ALDH<sup>+</sup> and ALDH<sup>−</sup> H460 cell populations by β-escin

We analyzed ALDH1A1 mRNA expression by RT-PCR in lung tumors of mice treated with NNK and fed control or 500 ppm β-escin diets. Compared with control, there was a significant reduction in the expression of ALDH1A1 in mice fed β-escin (P < 0.05; Fig. 4C). To assess whether β-escin may eliminate lung cancer stem cells, we treated H460 cells containing ALDH<sup>+</sup> population of cells with different concentrations of β-escin from 0 to 40 μmol/L and then were incubated for 24 hours. β-Escin treatment (0–40 μmol/L) significantly decreased the proportion of ALDH<sup>+</sup> population of cells in H460 cell line (Fig. 5A). When subpopulations of ALDH<sup>+</sup> and ALDH<sup>−</sup> cells were treated with 20 μmol/L β-escin, we found that there was a significant...
induction of p21 expression in β-escin–treated cells as compared with their respective controls (Fig. 5B), irrespective of whether the cells were positive or negative for the ALDH stem cell marker. In the H460 cell line, β-escin (0–40 μmol/L) decreased the expression of ALDH1A1, RhoA, and Rock in a dose-dependent manner as compared with control (Fig. 5C).

Discussion

The results presented here suggest that β-escin may provide a significant protective effect against tobacco carcinogen–induced lung tumor formation in female A/J mice. This study is consistent with the earlier observations that support possible antitumorigenic effects of β-escin in *in vitro* and *in vivo* models (6–10). Previously, we showed that β-escin, at 250 and 500 ppm in the diet, inhibited colon carcinogen–induced preneoplastic foci in rat colon in a dose-dependent manner (6). Our current results show that β-escin provides significant inhibition (up to 65%, *P* < 0.0001) of lung adenocarcinoma incidence and decreased tumor volume in a well-established model of lung cancer (Fig. 2). Our observations suggest that in β-escin fed mice, there is significant delay of progression adenoma to adenocarcinoma. Moreover, our results suggest about 39% to 53% inhibition (adenoma + adenocarcinoma) in β-escin diet fed mice as compared to control diet fed mice. These results clearly suggest that some of the NNK-induced lung lesions may not even progress to adenomas that turn to adenocarcinomas. There is relatively less adenocarcinoma inhibition at 34-week stage than 17-week stage; suggesting even treatment groups several adenomas had progressed to adenocarcinomas.
ALDH1A1 expression in mice fed 500 ppm β-escin, and activity in human lung H460 cancer cells was inhibited by β-escin, as shown with an Aldefluor assay (Fig. 5A). The suppression of ALDH+ cells in the well-established NNK-induced mouse lung tumor model, which has been used in lung cancer chemoprevention studies (23–27), and in vitro in the H460 human lung cancer cell line support the elimination of lung tumor CSCs by β-escin.

Previous studies have shown that the PCNA labeling index is high in human lung tumors and in the bronchial epithelial cells of smokers (28, 29). Dietary β-escin decreased the size of the lung tumors in mice as well as the PCNA labeling index as compared with those in mice fed control diet (Fig. 3). Cells commonly commit to proliferate or to differentiate in the G1 phase of the cell cycle, so we examined several signaling components involved in cell-cycle progression and tumorigenesis. In humans, loss of expression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 correlates with poor prognosis in lung cancer (30, 31). Patients with tumors who expressed p21 had a greater response to systemic chemotherapy (32) at some point during the course of cancer treatment. In mouse models studies, p21−/− mice showed an increased incidence of spontaneous lung tumors compared with p21-sufficient mice (33). We previously noted induction of p21WAF1/CIP1 and growth arrest by β-escin in colon cancer cell lines (6). We found that β-escin similarly induces p21 in NNK-induced mouse lung tumors and in ALDH+ as well as...
in ALDH\(^+\) H460 cells, arguing for effects of β-escin on signaling pathways independent of ALDH in addition to its ALDH inhibition (Figs. 3C, 4A and B, 5B and 5C).

Many reports indicate that p-Akt kinase, a downstream mediator of phosphatidylinositol 3-kinase (PI3K) signaling, may contribute to proliferation of NSCLC through activation of mTOR (34, 35). p-Akt plays a central role in processes of tumorigenesis (36), and its levels have prognostic value in lung cancer (37). The retinoic acid receptor is a direct substrate of Akt (38) and cyclin-dependent kinase (39, 40). A primary role of ALDH1A1 is to catalyze the conversion of retinol to retinoic acid (RA; refs. 17, 41), which binds RAR\(_{a}\), thereby trans-activating RA target genes. Previous reports have suggested that inhibition of ALDH1A1 activity leads to depletion of cellular RA, which, in most hematopoietic stem cells, delays the differentiation (42) by delaying exit from the cell-cycle exit (43) and fostering continued proliferation. Both ALDH activity and p-Akt were reduced significantly upon exposure to β-escin in lung tumor cells (Figs. 3 and 4). Thus, the ability of β-escin to interfere with both RA production and activation of the RAR may cause ALDH\(^+\) CSCs to exit the cell cycle and get arrested in G\(_1\) to S-phase, causing them to differentiate instead of to proliferate. These effects may contribute to the decrease in CSCs and lung tumor size by β-escin.

RA is essential in inducing RhoA and in its binding to Rock and activation of Rock kinase activity (44–46). Members of the Rho family of small GTP-binding proteins are key regulators of cytoskeletal changes, cell motility, and cell invasion (47). By cycling between active GTP-bound and inactive GDP-bound forms, RhoA acts as a molecular switch in regulating cytoskeleton formation and cell migration (48, 49). We observed that β-escin inhibits expression of ALDH1A1 and of RhoA, Rock, and β-actin followed by appropriate oxidase-conjugated secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.

Figure 5. Effects of β-escin on human H460 lung cancer cells. A, flow cytometric analysis of ALDH activity in H460 lung cancer cell line using the Aldefluor assay. Baseline fluorescence was established by inhibiting ALDH activity with DEAB (left) and used to generate a gate to identify ALDH\(^+\) cells among the lung cancer cells that have not been incubated with DEAB but have been incubated with different concentrations of β-escin. We observed a gradual decrease in the population of ALDH\(^+\) cells. B, effects of β-escin (20 µmol/L) on p21 expression as determined by conventional PCR in separate ALDH\(^+\) and ALDH\(^-\) populations of H460 cells as compared with control. C, concentration dependence for effect of β-escin on expression of ALDH1A1, p21, RhoA, Rock, and β-actin proteins was assessed by immunoblotting of H460 cells. SDS-PAGE and Western blotting were carried out with H460 lysates as described in Materials and Methods. Membranes were probed with specific primary antibodies for p21,cip1, RhoA, Rock, and β-actin followed by appropriate peroxidase-conjugated secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.
mechanisms for decreases in RhoA and Rock and the biologic significance need further investigation.

In summary, these data establish the chemopreventive efficacy of β-escin on NNK-induced lung carcinogenesis in the A/J mouse strain. β-escin was able to inhibit lung adenocarcinoma multiplicity and tumor size whether mice were short- or long-term exposed. β-escin inhibits ALDH1A1, and p-Akt and was able to induce p21 expression in lung tumor cells, suggesting growth arrest of tumor cells and CSCs as a possible mechanism of action.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.M.R. Patlolla, C.V. Rao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M.R. Patlolla, L. Biddick, Y. Zhang, D. Desai, C.V. Rao

References

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M.R. Patlolla, S.A. Lightfoot, C.V. Rao
Writing, review and/or revision of the manuscript: J.M.R. Patlolla, C.V. Rao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Qian, D. Desai, S. Amin, C.V. Rao

Study supervision: J.M.R. Patlolla, C.V. Rao

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Jagan M.R. Patlolla, Li Qian, Laura Biddick, et al.


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