Silibinin Protects against Photocarcinogenesis via Modulation of Cell Cycle Regulators, Mitogen-Activated Protein Kinases, and Akt Signaling

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ABSTRACT

Here, we assessed the protective effect of silibinin on UVB-induced skin carcinogenesis in SKH-1 hairless mice. Topical application of silibinin before or immediately after UVB exposure or its dietary feeding resulted in a strong protection against photocarcinogenesis, in terms of tumor multiplicity (60–66%; $P < 0.001$), tumor volume per mouse (93–97%; $P < 0.001$) and tumor volume per tumor (80–91%; $P < 0.001$). Silibinin also moderately inhibited tumor incidence (5–15%; $P < 0.01$) and delayed tumor latency period (up to 4 weeks; $P < 0.01–0.001$). To investigate *in vivo* molecular mechanisms of silibinin efficacy, tumors and uninvolved skin from tumor-bearing mice were examined immunohistochemically for proliferation, p53, apoptosis, and activated caspase-3. Silibinin treatment showed a strong decrease ($P < 0.001$) in proliferating cell nuclear antigen-positive cells and an increase in p53-positive ($P < 0.005–0.001$), terminal deoxynucleotidyltransferase-mediated nick end labeling-positive ($P < 0.005–0.001$), and cleaved caspase-3–positive cells ($P < 0.001$). Western blot analysis of normal skin and tumor lysates showed that silibinin decreases the levels of cyclin-dependent kinase 2 and cyclin-dependent kinase 4 and associated cyclins A, E, and D1, together with an up-regulation of Cip1/p21, Kip1/p27, and p53. Silibinin also showed a strong phosphorylation of extracellular signal-regulated protein kinase 1/2, stress-activated protein kinase/c-JUN NH$_2$-terminal kinase 1/2, and p38 mitogen-activated protein kinases but inhibited Akt phosphorylation and decreased survivin levels with an increase in cleaved caspase-3. Together, these results show a strong preventive efficacy of silibinin against photocarcinogenesis, which involves the inhibition of DNA synthesis, cell proliferation, and cell cycle progression and an induction of apoptosis. Furthermore, these results also identify *in vivo* molecular mechanisms of silibinin efficacy against photocarcinogenesis.

INTRODUCTION

More than one million new cases of nonmelanoma skin cancers including basal cell carcinoma and squamous cell carcinoma are diagnosed annually in the United States, resulting mostly from repeated sunlight exposure (1), in which UVB radiation is the causal etiologic factor (2). The first step in UVB-induced carcinogenesis involves induction of DNA damage via cyclobutane pyrimidine dimers and pyrimidine [6-4] pyrimidone photoproducts (2). DNA damage triggers a rapid increase in p53 that enhances Cip1/p21 synthesis and shuts off cell replication and DNA synthesis, allowing more time for DNA repair and/or apoptotic death of cells carrying damaged DNA (3). Unrepaired DNA damage, however, disrupts cellular processes finally introducing wrong bases into DNA, which results in mutations leading to loss or inappropriate expression of affected genes (4). UVB-induced initiated cells follow multiple additional hits by repeated UVB exposure leading to benign followed by malignant skin tumors in which clonal expansion of initiated cells involves oxidant–antioxidant imbalance, mitogenic and anti-apoptotic signaling cascades, and altered cell cycle and apoptosis regulation (5).

Together, these observations suggest that inhibiting UVB-caused alterations in skin might be useful in preventing nonmelanoma skin cancers. Several studies have shown that various phytochemicals protect against UVB-induced skin damages and cancer (5–7). Silymarin (flavonoid from milk thistle) and silibinin (bioactive component in silymarin) also cause strong prevention against UVB skin damages and photocarcinogenesis (8, 9). Furthermore, silibinin exerts dual efficacy in protecting or enhancing apoptosis in HaCaT cells depending on UVB damage, suggesting that it works as a UVB-damage sensor in deciding cell fate in its efficacy (10). Here, we report detailed mechanism-based efficacy studies with silibinin in UVB-induced skin tumorigenesis model. Photoprotective effects of silibinin revealed potential antitumor mechanisms involving inhibition of DNA synthesis and cell proliferation and apoptosis induction accompanied by modulation of p53 and cyclin-dependent kinase (CDK)-cyclin–CDK inhibitor levels. Additionally, alterations in mitogen-activated protein kinases (MAPKs) and Akt signaling, survivin expression, and caspase-3 activation were associated with the preventive efficacy of silibinin against photocarcinogenesis.

MATERIALS AND METHODS

Experimental Design. Silibinin diet (1%, w/w) was prepared by Dyets, Inc. (Bethlehem, PA) Female SKH-1 hairless mice (5 weeks old) were from Charles River Laboratories (Wilmington, MA). The UVB light source was four FS-40-T-12-UVB sunlamps with UVB Spectra 305 Dosimeter (Daavlin Co., Bryan, OH) emitting 80% radiation within 280 to 340 nm with a peak at 314 nm monitored with SEL 240 photodetector, 103 filter, and 1008 diffuser attached to IL1400A Research Radiometer (8, 9).

A long-term study was conducted assessing silibinin efficacy on UVB (180 mJ/cm^2 5 d/week for 25 weeks)-caused skin tumorigenesis and associated molecular events in female SKH-1 hairless mice. With or without UVB groups had 20 or 10 mice, respectively. Treatments were (*a*) unexposed/untreated control, (*b*) 9 mg of topical silibinin per 200 μL of acetone per mouse, (*c*) 180 mJ/cm^2 UVB five times per week for 25 weeks, (*d*) 9 mg of topical silibinin per 200 μL of acetone per mouse 30 minutes before UVB, (*e*) 9 mg of topical silibinin per 200 μL of acetone per mouse immediately after UVB, (*f*) 1% silibinin in diet and UVB for 25 weeks, and (*g*) 1% silibinin in diet for 25 weeks. Body weight and diet consumption were recorded throughout experiment, and for tumor study, data were analyzed for (*a*) percent tumor incidence, (*b*) tumor multiplicity (number per group), and (*c*) tumor volume per mouse (8). At experiment termination, tumors and uninvolved skin (from tumor-bearing mice) and normal skin (controls) were collected and used for immunohistochemical and/or Western analyses.

Bromodeoxyuridine Incorporation. Animals received injections i.p. of bromodeoxyuridine (BrdUrd; 50 mg/kg) and sacrificed 1 hour later; tissues were collected and processed; and sections were made. Endogenous peroxidase was blocked by 5% hydrogen peroxide in methanol for 10 minutes followed by incubation with 1 N HCl for 30 minutes at 37°C. Sections were incubated with mouse monoclonal anti-BrdUrd antibody (Sigma) for 2 h, rinsed with PBS, and incubated with conjugated horseradish peroxidase–streptavidin for 30 minutes at room temperature. Sections were then incubated with 3,3'-diaminobenzidine working solution for 10 minutes at room temperature, counterstained with diluted Harris hematoxylin, dehydrated, and mounted.

Immunostaining. Tissues were fixed in 10% formalin for 8 to 10 hours at 4°C, dehydrated in ethanol, cleared in xylene, and embedded in PolyFin. Four-μm serial sections were cut, processed, and immunostained using monoclonal proliferating cell nuclear antigen (PCNA; Dako, Carpinteria, CA), p53

(Novocastra Laboratories, Newcastle Upon Tyne, UK), and cleaved caspase-3 (Cell Signaling, Beverly, MA) antibodies (9). For negative staining controls, N-Universal Negative Control-mouse or rabbit antibody (Dako) was used under identical conditions. Terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) staining for apoptotic cells was done as published recently (9). All immunohistochemical analyses were done using Zeiss Axioscop 2 microscope (Carl Zeiss, Inc., Jena, Germany).

Lysate Preparation and Immunoblotting. Tissues were homogenized in lysis buffer, and lysates were prepared as published (10). Western immunoblotting was performed to probe cyclin A (H-432); cyclin E (C-19); cyclin D1 (M-20); CDK2 (M-2); CDK4 (H-303; Santa Cruz Biotechnology, Santa Cruz, CA); p53 (Novocastra Laboratories); survivin (Novus, Littleton, CO); phospho- and total extracellular signal-regulated protein kinase (ERK) 1/2, c-JUN NH$_2$-terminal kinase (JNK) 1/2, p38, and Akt; cleaved caspase-3 (Cell Signaling); and Cip1/p21 (Upstate), Kip1/p27 (Ab-1, Clone DCS-72.F6), and tubulin-α (Ab-2; Lab Vision, Fremont, CA) as published recently (10).

Statistical Analysis. Statistical significance of difference between UVB alone *versus* all other groups was determined by one-way ANOVA followed by Bonferroni t test using SigmaStat 2.03. $P < 0.05$ was considered statistically significant.

RESULTS

Protective Effects of Topical Application/Dietary Feeding of Silibinin on Ultraviolet B-Induced Tumorigenesis. To evaluate silibinin efficacy against UVB-induced tumorigenesis, three different protocols were selected in which it was applied topically (*a*) before or (*b*) immediately after UVB or (*c*) fed in diet. UVB exposure of mice at 180 mJ/cm^2 dose 5 d/week caused 100% tumor incidence at 25 weeks; however, silibinin treatments before or immediately after UVB or its dietary feeding resulted in 85 to 95% incidence accounting for moderate (5–15%) protection (Fig. 1*A*). In terms of latency period (appearance of first tumor), compared with UVB group showing tumor appearance at 12 weeks, silibinin pre- or post-topical application or its dietary feeding showed first tumor appearance at 16, 14, and

15 weeks ($P < 0.01$–0.001), accounting for a delay in latency period by 4, 2, and 3 weeks, respectively (Fig. 1*A*).

Whereas silibinin efficacy toward tumor incidence and latency was moderate, it showed very strong effects on tumor multiplicity and volume. Compared with UVB alone, silibinin treatments in three different protocols resulted in a strong reduction in total number of tumors per group throughout experiment (Fig. 1*B*). At 25 weeks, compared with UVB group with a total of 273 tumors, number of tumors per group in silibinin pre- or post-topically treated or dietary-fed group were 92, 106, and 108, accounting for 66, 61, and 60% inhibition ($P < 0.001$), respectively (Fig. 1*B*). Similarly, in terms of number of tumors per mouse, compared with UVB group with 13.65 ± 1.48 tumors, silibinin topical treatment in pre- or post-UVB or dietary feeding showed 4.6 ± 0.64, 5.3 ± 0.55, and 5.4 ± 0.83 tumors/mouse accounting for 60 to 66% protection ($P < 0.001$), respectively (data not shown). In case of tumor volume, compared with UVB group with 2765 ± 759 mm^3 tumor volume/mouse, total tumor volume per mouse in pre- or post-silibinin topical treatment or dietary feeding group was only 92 ± 19, 98 ± 17, and 200 ± 53 mm^3 accounting for 93 to 97% inhibition ($P < 0.001$), respectively (Fig. 1*C*). Similarly, when data were analyzed for tumor volume per tumor, compared with UVB group with 188 ± 50 mm^3 volume/tumor, silibinin pre- or post-topical treatment or dietary feeding resulted in only 16 ± 3, 17 ± 2, and 40 ± 15 mm^3 volume/tumor, respectively, accounting for 80 to 91% ($P < 0.001$) inhibition (data not shown). Overall, our completed tumorigenesis experiment clearly and convincingly shows a strong protective effect of topically applied (before or immediately after UVB) and dietary-fed silibinin against UVB-induced tumorigenesis in mouse skin.

To assess whether silibinin supplementation affects diet consumption, diet consumption profiles were followed for control groups of mice on regular or 1% silibinin supplemented (w/w) diet for 25 weeks, a time-period identical to experimental groups. Compared with regu-
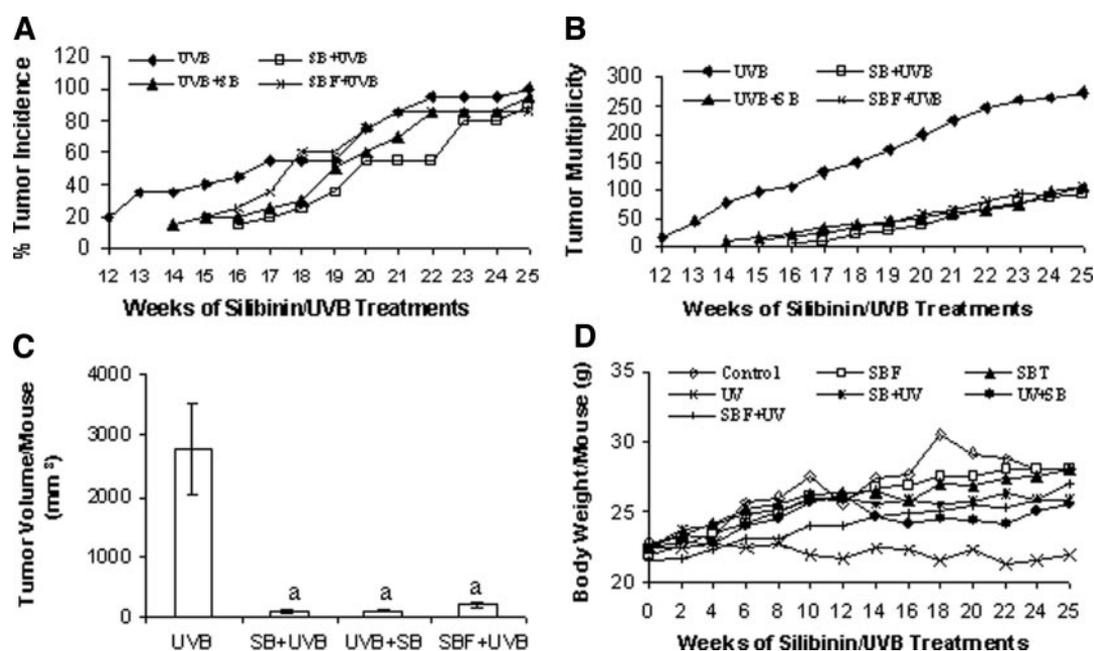
Fig. 1. Effect of topical application/dietary feeding of silibinin on UVB-induced tumorigenesis and body weight gain profiles. SKH-1 hairless mice were unexposed (*control*), exposed with UVB (180 mJ/cm^2) alone (*UVB*), topically applied with 9 mg of silibinin in 200 μL of acetone per mouse and exposed to UVB (post- and pre-irradiation, *SB+UVB* and *UVB+SB*, respectively), or fed with 1% silibinin in diet (w/w) throughout the experiment and exposed to UVB (*SBF+UVB*) as detailed in Materials and Methods. Percent tumor incidence (*A*) and tumor multiplicity (*B*) were recorded throughout the experiment. *C*, Tumor volume per mouse (mm^3) data shown are at the end of the experiment at 25 weeks. In each case, the data shown are from 20 mice in each group; the data shown in *C* are mean ± SE (*bars*) of 20 mice in each group. No tumors were observed in control and dietary or topically treated silibinin alone groups, and therefore, these data are not shown. *D*, Body weight per mouse (g) was also recorded throughout the experiment. The statistical significance of difference between UVB-treated and all other groups was determined by one-way ANOVA followed by Bonferroni t test for multiple comparisons. *SB*, silibinin; *SBT*, silibinin applied topically; *SBF*, silibinin fed in diet. *a*, $P \le 0.001$.

lar diet, animals in silibinin dietary supplement group consumed diet at a much higher level throughout 25 weeks of study (data not shown). Body weight gain profile is one of the most extensively used measures for an overall systemic toxicity and untoward side effects in cancer chemoprevention (11). Accordingly, we also recorded body weight for each mouse in all control and experimental groups. Neither topical nor dietary silibinin showed any noticeable change in body weight gain profile throughout study when compared with controls. Conversely, animals in UVB alone group showed a strong decrease in body weight with increasing weeks of UVB when compared with those in (*a*) control, (*b*) silibinin treatments in two different protocols, and (*c*) silibinin treatments in three different protocols and UVB (Fig. 1*D*). Together, these observations suggest that dietary silibinin is well accepted by mice in terms of food consumption and that topical and dietary silibinin treatments do not exert toxicity or adverse health effects in mice.

Silibinin Inhibits Bromodeoxyuridine Incorporation in Epidermal Cells. To evaluate proliferating cells predominantly in S phase, we measured BrdUrd incorporation into the DNA of epidermal cells (Fig. 2*A*). UVB irradiation for 25 weeks showed strong immunoreactivity for BrdUrd in uninvolved skin (data not shown) accounting for $23.2 \pm 2.2\%$ BrdUrd-positive cells compared with 5.3 ± 1.5, 4.8 ± 2.5, and $7.1 \pm 1.5\%$ in untreated, dietary, and topical silibinin–treated but unirradiated groups (4.4-fold increase in UVB alone *versus* unirradiated group, $P < 0.001$), respectively (Fig. 2*A*). Topical application of silibinin before or immediately after UVB or its dietary feeding, however, resulted in $11.8 \pm 1.1\%$ ($P < 0.001$), $14.9 \pm 1.2\%$ ($P < 0.001$), and $16.9 \pm 1.1\%$ ($P < 0.005$) BrdUrd-positive cells in uninvolved skin, accounting for 49, 35, and 27% decrease, respectively (Fig. 2*A*), suggesting that silibinin inhibits UVB-induced DNA synthesis in mouse skin epidermal cells.

Silibinin Inhibits Cell Proliferation in Ultraviolet B-Induced Tumorigenesis. To further determine whether topical or dietary silibinin treatment affects UVB-induced cell proliferation, uninvolved skin and tumor samples and respective unirradiated skin samples were evaluated for PCNA immunostaining (data not shown). In unirradiated mice receiving normal diet, $4.2 \pm 1.5\%$ PCNA-positive cells were observed in skin that were comparable with those in topically (4.3 ± 0.6) or dietary (4.8 ± 1.5) silibinin alone group (Fig. 2*B*). A strong PCNA immunostaining, however, was observed in tumors and epidermis from uninvolved skin in UVB alone group (data not shown), accounting for $38.8 \pm 1.6\%$ and $21.6 \pm 1.4\%$ PCNA-positive cells ($P < 0.001$ *versus* unirradiated controls), respectively (Fig. 2*B* and *C*). Silibinin treatment before or immediately after UVB or its dietary feeding decreased PCNA-positive cells to $16.3 \pm 1.4\%$ ($P < 0.001$), $18.0 \pm 1.1\%$ ($P < 0.001$), and $15.7 \pm 1.2\%$ ($P < 0.005$) in tumor samples, accounting for 54 to 60% decrease (Fig. 2*C*). Silibinin treatment also decreased (12–36%), although to a lesser degree, PCNA-positive cells to $16.2 \pm 1.8\%$ ($P < 0.001$), $18.9 \pm 1.7\%$ ($P < 0.001$), and $13.8 \pm 1.1\%$ ($P < 0.001$) in uninvolved skin from tumor-bearing mice, respectively (Fig. 2*B*).

Silibinin Up-regulates p53 Protein Level in Ultraviolet B-Induced Tumorigenesis. p53 is known to play a key role in cell cycle regulation and apoptosis (12), and therefore, next, we analyzed its expression by immunostaining (data not shown) in tumor and uninvolved skin samples. Exposure of mice to multiple UVB irradiation in 25 weeks tumorigenesis protocol resulted in $14.7 \pm 1.7\%$ ($P < 0.001$) and $11.2 \pm 1.1\%$ ($P < 0.001$) p53-positive cells in tumor and uninvolved skin sections, respectively, compared with $1.8 \pm 1.1\%$ in unirradiated controls (Fig. 3*A* and *B*). Silibinin treatment before or immediately after UVB or its dietary feeding, however, increased p53-positive cells to $31.8 \pm 1.2\%$ ($P < 0.001$), $24.2 \pm 1.5\%$ ($P < 0.005$), and $32.0 \pm 2.2\%$ ($P < 0.005$) in the
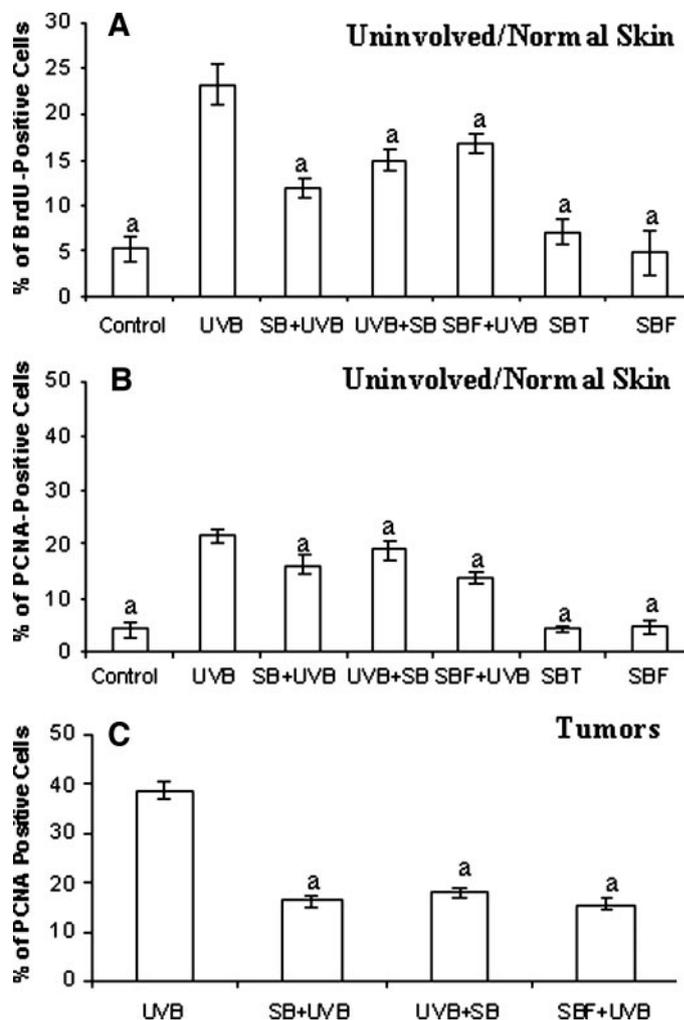

Fig. 2. Effect of silibinin on BrdUrd incorporation in epidermal cells and proliferation in UVB-induced tumorigenesis. Treatment regimens are those mentioned in Fig. 1, and other details are described in Materials and Methods. Percent of BrdUrd-positive cells in uninvolved skin from tumor-bearing mice and in normal skin from respective control groups (*A*), percent of PCNA-positive cells in uninvolved skin from tumor-bearing mice and in normal skin from respective control groups (*B*), and percent of PCNA-positive cells in tumors (*C*) were recorded by counting the BrdUrd- or PCNA-stained cells over total cells in randomly selected fields ($\times 400$) from each tissue sample. Data shown are mean $\pm$ SE of 15 to 25 fields per five mice per group. The statistical significance of difference between UVB-treated and all other groups was determined by one-way ANOVA followed by Bonferroni *t* test for multiple comparisons. *SB*, silibinin; *SBT*, silibinin applied topically; *SBF*, silibinin fed in diet. *a*, $P \leq 0.001$; *b*, $P \leq 0.005$.

tumors, respectively, accounting for 1.7- to 2.3-fold increase over UVB alone tumors (Fig. 3*A*). Similarly, silibinin showed an increase in p53-positive cells to $19.8 \pm 1.7\%$ ($P < 0.001$), $17.5 \pm 1.8\%$ ($P < 0.005$), and $17.1 \pm 1.9\%$ ($P < 0.005$) in uninvolved skin, respectively, accounting for 1.5- to 1.8-fold increase over UVB alone group (Fig. 3*B*). In other control groups, dietary or topical silibinin alone, without UVB, did not show any change in p53 positive cells in the skin when compared with unirradiated controls (Fig. 3*B*).

Silibinin Increases Apoptosis in Ultraviolet B-Induced Tumorigenesis. DNA is the primary target of UVB damage, causing either apoptotic death of damaged cells or fixation of mutation in p53 leading to tumor initiation (4). p53 accumulation after DNA damage by UVB plays an important role in DNA repair and apoptotic death of damaged cells (4). Based on our results showing that tumors and uninvolved skin from tumor-bearing mice in three different silibinin treatment protocols have significantly higher levels of p53 compared with UVB alone, we next assessed percentage apoptotic cells by TUNEL staining (data not shown). Quantitative analyses of TUNEL-
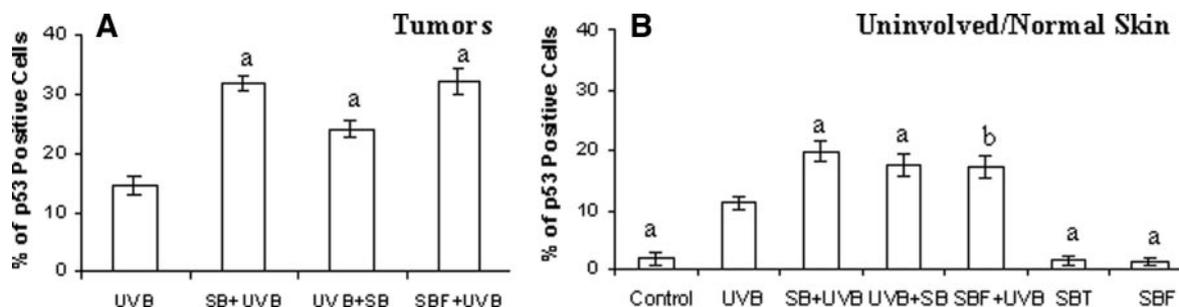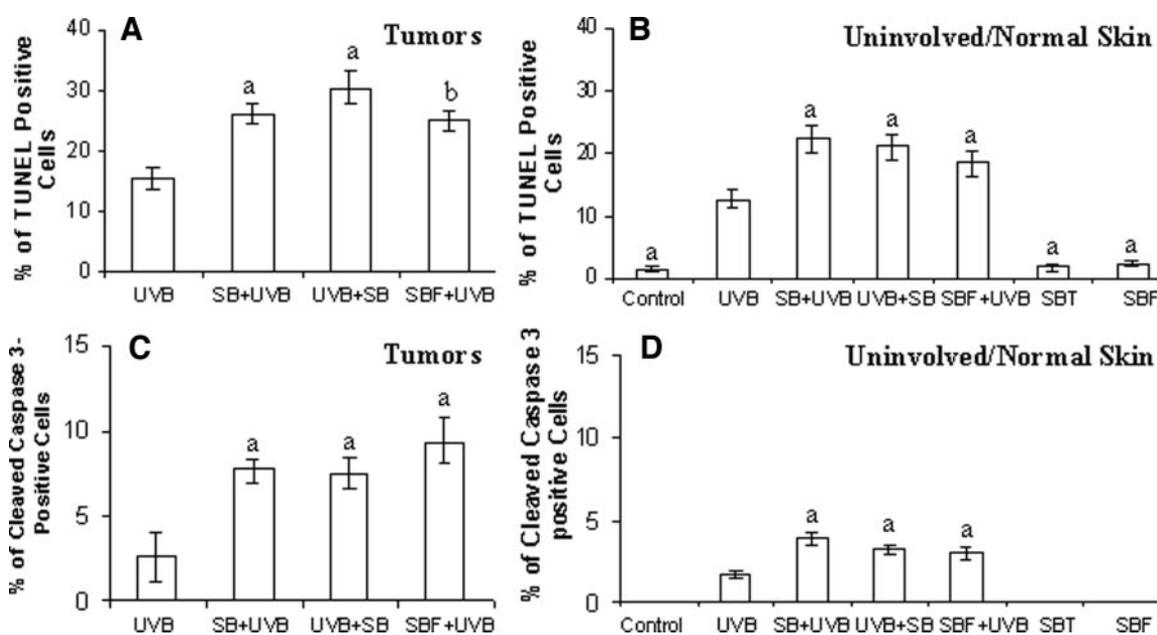
Fig. 3. Effect of silibinin on p53 protein levels in UVB-induced tumorigenesis. Treatment regimens are those mentioned in Fig. 1, and other details are described in Materials and Methods. Percent of p53-positive cells in uninvolved skin from tumor-bearing mice and in normal skin from respective control groups (*A*) and percent of p53-positive cells in tumors (*B*) were recorded by counting the p53-stained cells over total cells in randomly selected fields (×400) from each tissue sample. Data shown are mean ± SE of 15 to 25 fields per five mice per group. The statistical significance of difference between UVB-treated and all other groups was determined by one-way ANOVA followed by Bonferroni *t* test for multiple comparisons. *SB*, silibinin; *SBT*, silibinin applied topically; *SBF*, silibinin fed in diet. *a*, $P \leq 0.001$; *b*, $P \leq 0.005$.

positive cells showed 15.4 ± 1.7% (11-fold, $P < 0.001$) and 12.7 ± 1.4% (9-fold, $P < 0.001$) apoptotic cells in tumor and uninvolved skin samples, respectively, from UVB alone group compared with 1.4 ± 0.3% in unirradiated controls (Fig. 4*A* and *B*). However, silibinin treatment before or immediately after UVB or its dietary feeding further increased the TUNEL-positive cells to 26.1 ± 1.8% ($P < 0.001$), 30.3 ± 2.6% ($P < 0.001$), and 24.9 ± 1.5% ($P < 0.001$) in tumor samples, respectively, accounting for 1.6- to 2.0-fold increase over UVB alone tumors (Fig. 4*A*). Similar silibinin treatments also resulted in an increase in TUNEL-positive cells to 22.2 ± 2.2% ($P < 0.001$), 20.9 ± 2.2% ($P < 0.001$), and 18.4 ± 2.1% ($P < 0.001$) in uninvolved skin, respectively, which accounted for 1.4- to 1.7-fold increase compared with UVB alone (Fig. 4*B*). Together, these results suggested the apoptotic potential of silibinin in chronic UVB-exposed skin as well as tumors.

Silibinin Up-regulates Cleaved Caspase-3 Level in Ultraviolet B-Induced Tumorigenesis. To assess possible involvement of caspase activation in silibinin-caused increase in apoptosis, next we performed cleaved caspase-3 immunostaining in tumors and uninvolved skin samples from tumor-bearing mice and respective unirra-

diated control skin samples (data not shown). Quantitative analyses of immunostaining revealed that topical application of silibinin before or immediately after UVB, or its dietary feeding for 25 weeks increases percent of cleaved caspase-3–positive cells by 2.9- to 3.6-fold ($P < 0.001$) in tumor and 1.7- to 2.2-fold ($P < 0.001$) in uninvolved skin samples compared with UVB alone group (Fig. 4*C* and *D*). Exposure of mice to UVB (180 mJ/cm² 5 d/week for 25 weeks) resulted in 2.6 ± 1.4% and 1.8 ± 0.2% cleaved caspase-3–positive cells in tumor and uninvolved skin sections, respectively (Fig. 4*C* and *D*), with no detectable cleaved caspase-3 staining in unirradiated controls without or with silibinin treatment (Fig. 4*D*). However, silibinin treatment in three different protocols increased caspase-3–positive cells to 7.7 ± 0.7% ($P < 0.001$), 7.5 ± 0.9% ($P < 0.01$), and 9.4 ± 1.4% ($P < 0.005$) in tumor sections (Fig. 4*C*) and to 3.9 ± 0.4% ($P < 0.001$), 3.2 ± 0.3% ($P < 0.001$), and 3.0 ± 0.4% ($P < 0.001$) in uninvolved skin sections, respectively (Fig. 4*D*).

Silibinin Modulates Cell Cycle Regulators in Ultraviolet B-Induced Tumorigenesis. Enhanced expression of cell cycle regulatory proteins such as CDKs and cyclins or decreased expression of CDK inhibitors have been implicated in photocarcinogenesis (13).

Fig. 4. Effect of silibinin on apoptosis in UVB-induced tumorigenesis. Treatment regimens are those mentioned in Fig. 1, and other details are described in Materials and Methods. Percent of TUNEL- and cleaved caspase 3-positive cells in tumors (*A* and *C*), and percent of TUNEL- and cleaved caspase 3-positive cells in uninvolved skin from tumor-bearing mice and in normal skin from respective control groups (*B* and *D*), respectively, were recorded by counting the TUNEL or cleaved caspase 3-stained cells over total cells in randomly selected fields (×400) from each tissue sample. Data shown are mean ± SE of 15 to 25 fields five mice per group. The statistical significance of difference between UVB-treated and all other groups was determined by one-way ANOVA followed by Bonferroni *t* test for multiple comparisons. *SB*, silibinin; *SBT*, silibinin applied topically; *SBF*, silibinin fed in diet. *a*, $P \leq 0.001$; *b*, $P \leq 0.005$.
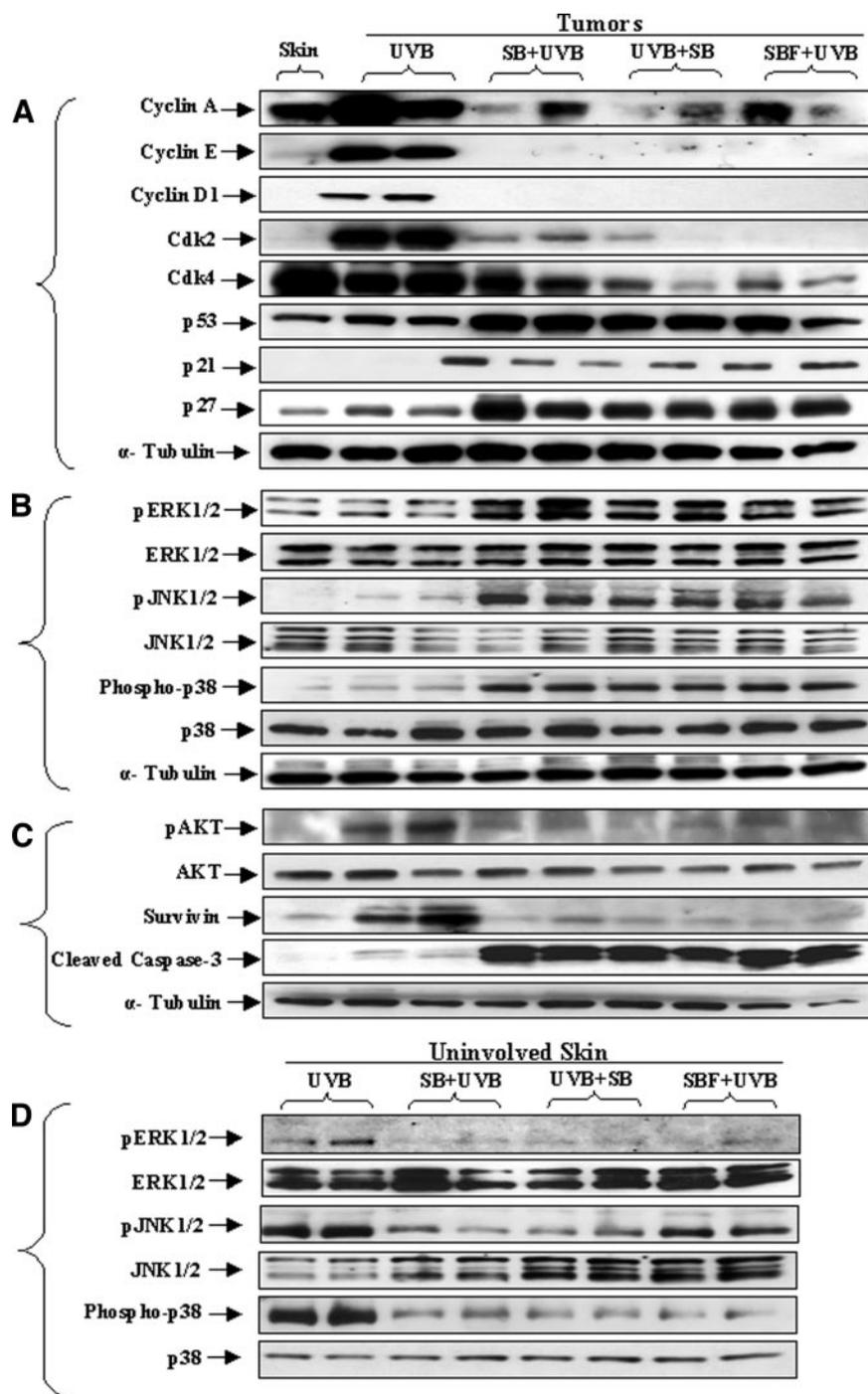
Fig. 5. Effect of silibinin on cell cycle regulators; MAPK activation cascades; and phospho-Akt, survivin, and cleaved caspase-3 levels in UVB-induced tumorigenesis. Normal skin (unirradiated control) and tumor and uninvolved skin (irradiated) samples from the long-term tumorigenesis experiment (as labeled in the figure) were homogenized in lysis buffer, and lysates were subjected to SDS-PAGE followed by Western blotting, as described in Materials and Methods. The membranes were probed for cell cycle regulators (A), MAPK members (B and D), and phospho- and total Akt, survivin, and cleaved caspase-3 (C), employing specific antibodies detailed in Materials and Methods and then peroxidase-conjugated appropriate secondary antibody. Proteins were visualized with enhanced chemiluminescence detection system. Membranes were stripped and reprobed with α-tubulin antibody for protein loading correction.

Based on our results showing an inhibition of tumor growth and cell proliferation together with an increase in p53 expression by silibinin in UVB-induced tumorigenesis protocols, we rationalized that silibinin might have modulatory effects on cell cycle regulatory proteins. As shown in Fig. 5A, compared with normal skin from age-matched unirradiated controls, tumor tissues from UVB alone group showed a strong increase in cyclins (cyclin A, E, and D1) and CDK2 levels; protein expression of CDK4 was reduced considerably in tumors compared with normal skin. However, we observed a strong decrease in cyclin A, cyclin E, cyclin D1, CDK2, and CDK4 levels in tumors harvested from the groups of mice treated with silibinin before or immediately after UVB, or its dietary feeding compared with UVB alone tumors (Fig. 5A). Next, we examined levels of Cip1/p21, which

is a universal inhibitor of cell cycle progression and is transcriptionally activated by p53 after UVB (12, 14). Consistent with silibinin effect in up-regulating p53 protein levels, we observed an increase in Cip1/p21 in silibinin-UVB–treated groups of tumors compared with UVB alone (Fig. 5A). Kip1/p27 is another important CDK inhibitor that regulates CDK–cyclin activity at G$_1$–S transition (14). Protein levels of Kip1/p27 were also strongly up-regulated in silibinin-UVB–treated groups of tumors compared with those in UVB alone group (Fig. 5A). In the studies assessing p53 levels by immunoblotting, consistent with immunostaining data, compared with unirradiated (normal) skin, tumor samples from UVB alone group showed strong levels of p53 protein; however, tumors from silibinin-UVB–treated groups of mice showed much stronger levels of p53 (Fig. 5A). Rep-
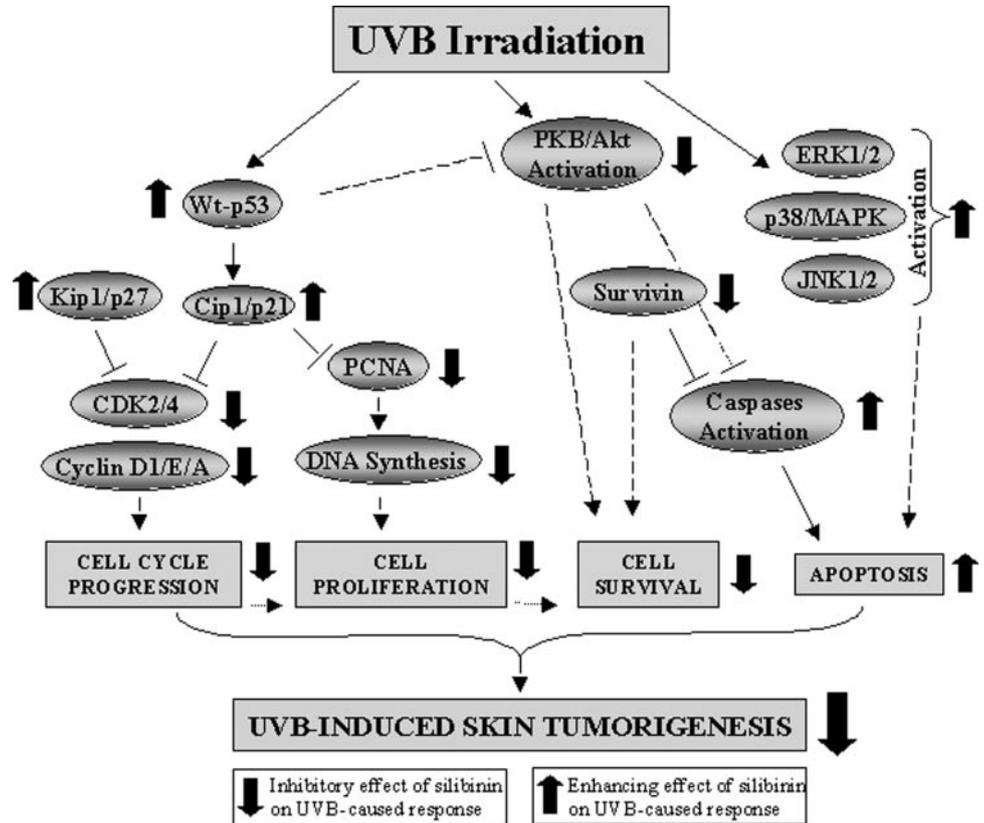
Fig. 6. Schematic presentation of the proposed molecular mechanisms of protective effects of silibinin against UVB-induced skin carcinogenesis.

mechanism from mutagenesis/carcinogenesis. Consistent with these reports, we observed a strong association between an increased levels of p53 and apoptosis induction in silibinin-treated chronic UVB exposed skin and tumors compared with UVB alone samples. Interestingly, these results are in contrast with another recent study by us showing that in an acute single UVB exposure protocol, silibinin treatments under identical conditions cause an up-regulation of p53 protein but protect cells from apoptotic death (9). Together, the results of present study and those published recently by us (9), clearly demonstrate "dual efficacy" of silibinin in protecting cells against UVB-induced apoptosis if damage is acute (9) and enhancing apoptosis if UVB-induced damage is chronic; however, both situations involve p53 up-regulation by silibinin as reported by Tron *et al.* (31).

Regulation of cyclin–CDK complexes plays a key role in cell cycle progression at different phases in which CDKs are negatively regulated by a group of functionally related proteins known as CDK inhibitors such as Kip/Cip family members (32, 33). Cip1/p21 is a universal CDK inhibitor and is mainly regulated by p53 (14). Cip1/p21 binds PCNA and inhibits PCNA function in DNA replication in a p53-dependent manner (34); Kip1/p27 is up-regulated in response to antiproliferative signals (35). Consistent with these reports, silibinin treatments resulted in an up-regulation of Kip1/p27 and Cip1/p21 and decreased CDK2, CDK4, cyclin E, cyclin A and cyclin D1 protein levels in tumors, which could possibly be associated with a cell cycle arrest in tumor cells causing a decrease in proliferation as observed by a decreased PCNA immunostaining. Overall, it could be suggested that inhibition in cell proliferation could be one of the major mechanisms of silibinin efficacy against UVB-induced tumorigenesis.

MAPKs are important upstream regulators of transcription factor activities, and their signaling is critical to transduction of a wide variety of extracellular stimuli into intracellular cascades, thereby controlling the cellular events such as proliferation, differentiation, and apoptosis (36). MAPK signaling cascades are important in regulating magnitude of

UV-induced cellular responses (15), in which ERK1/2 activation causes cell proliferation and survival, but JNK1/2 and p38 kinase seem to exert anti- and pro-apoptotic functions (18). ERK activation also exerts dual role where its very high intensity halts cell cycle by inducing CDK inhibitor Cip1/p21 and Kip1/27 expression (37). Ceramide, Taxol, and etoposide have very different intracellular targets yet induce apoptosis through ERK1/2 activation cascade in A431 cells; they also activate JNK1/2 and p38 kinases in their apoptotic response in cancer cells (18). JNK1/2 and p38 kinase also mediate various forms of cellular stress, such as damage repair mechanisms, cell growth arrest, and apoptotic cell death (38). P38 mediates UVB apoptosis by inducing cytochrome *c* release into cytosol followed by caspase-3 activation in HaCaT cells (39). Conversely, UVB-caused induction of p38 activity followed by activation of transcription factor AP-1 is also reported in HaCaT human keratinocytes, which could lead to pro-survival/anti-apoptotic response (40). Consistent with these reports, silibinin treatments in UVB tumorigenesis studies resulted in a strong increase in ERK1/2, JNK1/2, and p38 phosphorylation in tumor samples, suggesting their possible involvement in an overall silibinin efficacy in causing apoptosis induction in tumor cells.

Akt signaling plays a prominent role in several processes considered hallmarks of cancer (19). Akt makes a tumor cell overly responsive to ambient levels of growth factors for survival that normally would not provoke proliferation. Furthermore, Akt activation affects cell cycle progression, through regulation of cyclin D stability (41) and a decrease in Kip1/p27 via its phosphorylation and degradation (42). In apoptosis-prone cells, p53-dependent signaling also enables Akt down-regulation, predisposing them to rapid apoptosis in response to a combination of cellular stress and decreased survival signals (43). An activated p53 causes a rapid decrease in steady-state levels of Akt by its caspase-mediated degradation (44). Consistent with these reports, we observed an almost complete decrease in phospho-Akt in tumor samples from silibinin-UVB–treated groups compared with its strong levels in tumors from UVB alone group,

suggesting possible involvement of cross-talk between Akt and p53 in apoptosis regulation in tumor cells from silibinin treatment groups. Based on this association, it could be reasoned that silibinin-caused pronounced accumulation of p53 might also result in a down-regulation of Akt activation rendering cells more prone not only to direct apoptotic effects of p53, but also to all other concurrent apoptotic signals, outcomes of which are normally blocked by activated Akt.

Recent studies have explained and established molecular mechanism of apoptosis regulation by survivin via its interaction with caspases pathway (45). Molecular antagonists of survivin including antisense, ribozymes, short interferring RNA sequences, and dominant-negative mutants have been shown to cause caspase-dependent cell death, enhancement of apoptotic stimuli, and anticancer activity, *in vivo* (46, 47). Furthermore, a recent study shows that survivin expression in mouse skin prevents papilloma regression and promotes tumor progression (48). Consistent with these reports, silibinin treatment decreased survivin level in tumors that might have a direct role in caspase-3 activation causing apoptotic death of tumor cells in silibinin treatment groups as observed in present study. In addition, cell death induced through p53 pathway is also executed by caspase cascade, which is the central event in effector phase of apoptosis (49). In this regard, silibinin treatment strongly induced activated caspase-3 levels and the number of TUNEL-positive cells in tumor and uninvolved skin samples.

In summary, the findings in present study show that topical application of silibinin before or immediately after UVB irradiation or its dietary feeding to SKH-1 mice exposed to UVB, strongly inhibits UVB-induced tumorigenesis. Our data also suggest that observed preventive efficacy of silibinin is most likely mediated via an inhibition in DNA synthesis, cell proliferation, and cell cycle arrest and an induction of apoptosis. As summarized in Fig. 6, the molecular events identified to be associated with silibinin efficacy include (*a*) an increased accumulation of p53 protein, (*b*) an induction of CDK inhibitors together with a decrease in CDK–cyclin levels, (*c*) activation of MAPK pathways, (*d*) inhibition of Akt signaling, and (*e*) decreased level of survivin concomitant with caspase-3 activation.

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