Direct Targeting of MEK1/2 and RSK2 by Silybin Induces Cell-Cycle Arrest and Inhibits Melanoma Cell Growth

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Abstract
Abnormal functioning of multiple gene products underlies the neoplastic transformation of cells. Thus, chemopreventive and/or chemotherapeutic agents with multigene targets hold promise in the development of effective anticancer drugs. Silybin, a component of milk thistle, is a natural anticancer agent. In the present study, we investigated the effect of silybin on melanoma cell growth and elucidated its molecular targets. Our study revealed that silybin attenuated the growth of melanoma xenograft tumors in nude mice. Silybin inhibited the kinase activity of mitogen-activated protein kinase (MEK)-1/2 and ribosomal S6 kinase (RSK)-2 in melanoma cells. The direct binding of silybin with MEK1/2 and RSK2 was explored using a computational docking model. Treatment of melanoma cells with silybin attenuated the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and RSK2, which are regulated by the upstream kinases MEK1/2. The blockade of MEK1/2-ERK1/2-RSK2 signaling by silybin resulted in a reduced activation of NF-κB, activator protein-1, and STAT3, which are transcriptional regulators of a variety of proliferative genes in melanomas. Silybin, by blocking the activation of these transcription factors, induced cell-cycle arrest at the G1 phase and inhibited melanoma cell growth in vitro and in vivo. Taken together, silybin suppresses melanoma growth by directly targeting MEK- and RSK-mediated signaling pathways. Cancer Prev Res; 6(5); 455–65. ©2013 AACR.

Introduction
Skin cancer is the most common form of cancer in the United States (1). Melanoma, the most aggressive form of skin cancer, accounts for 75% of skin cancer mortality (2).

Although many common cancers are declining, the incidence of melanoma continues to rise at an estimated annual rate of 3.1% (2, 3). Malignant melanoma involves mutations in BRAF (V600E) and NRAS at approximately 60% and 20%, respectively (4, 5). Both the oncogenic BRAF and NRAS have a common pathway, mitogen-activated protein kinase kinase (MEK)-1/2, which is involved in the activation of extracellular signal-regulated kinase (ERK)-1/2 as the downstream signaling pathway (6, 7). The activation of the signaling cascade comprising BRAF-NRAS-MEK1/2-ERK1/2 is an important trigger for melanoma survival, growth, and proliferation. Therefore, the development of drugs targeting multiple components of this signaling pathway could reduce the incidence and mortality of melanoma skin cancer.

Silybin (also known as silibinin), a major bioactive component of milk thistle (Silybum marianum), has long been used for the prevention of allergies (8) and hepatic damage (9). Several studies have shown the chemopreventive and/or chemotherapeutic effects of silybin against various cancers, including those of the colon, prostate, bladder, and lung (10–16). However, a direct molecular target for this anticancer agent is yet to be identified. Virtual screening of a variety of U.S. Food and Drug Administration (FDA) drug/supplementary/integrative medicine lead databases revealed that silybin is a potent inhibitor of the BRAF-MEK-ERK-RSK2 signaling pathway. Inappropriate amplification of this signaling pathway leads to the activation of transcription factors, such as NF-κB, activator...
protein-1 (AP-1), and STAT3, which contribute to melanoma development and progression. In the present study, we report that silybin significantly inhibited the growth of the SK-MEL-5 melanoma cell line in vitro and in vivo through its direct binding with MEK1/2 and ribosomal S6 kinase (RSK)-2, resulting in the inhibition of their catalytic kinase activities.

Materials and Methods

Reagents
Silybin was purchased from LKT laboratories. Active BRAF (V600E), active MEK1, inactive ERK2 (MEK substrate), active RSK2, and inactive MEK human recombinant proteins for kinase assays were purchased from Millipore. The active MEK2 human recombinant protein for a kinase assay was purchased from Signal Chem. Antibodies to detect phosphorylated MEK (pMEK), total ERKs, phosphorylated ERKs (pERK), total RSK, phosphorylated RSK (pRSK), and cyclin D1 were purchased from Cell Signaling Technology. Antibodies against total MEK1, total MEK2, total MEK1/2, and β-actin were purchased from Santa Cruz Biotechnology.

Cell culture
The human melanoma, SK-MEL-5 and SK-MEL-28, cell lines were purchased from American Type Culture Collection (ATCC). The SK-MEL-2 cell line was kindly provided by Dr. S. Chellappan (National Functional Genomics Center H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL; ref. 17). SK-MEL-5 and SK-MEL-28 cells were cultured in minimum essential medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), sodium pyruvate (1 mM), and 10% FBS ( Gibco). SK-MEL-2 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (1:1) containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS. Cells were maintained at 5% CO2, 37°C in a humidified incubator. All cells were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks.

In vitro kinase assay
The kinase assay was conducted in accordance with instructions provided by Upstate Biotechnology. Briefly, the reaction was carried out in the presence of 10 μCi of [γ-32P] ATP with each compound in 20 μL of reaction buffer containing 20 mMol/L HEPES (pH 7.4), 10 mMol/L MgCl2, 10 mMol/L MnCl2, and 1 mMol/L dithiothreitol (DTT). After incubation at room temperature for 30 minutes, the reaction was stopped by adding 5 μL protein loading buffer, and the mixture was separated by SDS-PAGE. Each experiment was repeated twice and the relative amounts of incorporated radioactivity were assessed by autoradiography.

Computational modeling
The crystal structures of MEK1 and RSK2 used as the receptor structures were downloaded from the Protein Data Bank (PDB; ref. 18). The coordinates of silybin were downloaded from the PubChem compound database (19). Before ligand–protein docking, the raw PDB structures were converted into the all-atom, fully prepared receptor model structures by using the Protein Preparation Wizard module (20). The original 2-dimensional (2D) structure of silybin was changed to 3D conformers using ConGen (21). Protein–ligand docking was run using the high-performance hierarchical docking algorithm, Glide (22, 23). The final binding models of MEK1-silybin and RSK2-silybin were generated from Schrödinger Induced Fit Docking (IFD; ref. 24), which merges the predictive power of Prime with the docking and scoring capabilities of Glide for accommodating possible protein conformational changes upon ligand binding. More computational details are provided in Supplementary Materials and Methods.

Reporter gene activity
Transient transfection was conducted using jetPEI (Qbiogene), and luciferase reporter gene activity assays were conducted according to the manufacturer’s instructions (Promega). Cells (1 × 104 per well) were seeded the day before transfection into 12-well culture plates. Cells were cotransfected with the NF-kB, AP-1, and STAT3 reporter plasmid (800 ng) and an internal control (tk-Renilla, 50 ng) in 12-well plates and incubated for 24 hours. Melanoma cells were treated with vehicle or 40 or 80 μmol/L silybin for 9 hours. Cells were harvested in Promega lysis buffer. Firefly and Renilla luciferase activities were measured using substrates provided in the reporter assay system (Promega). The luciferase activity was normalized to Renilla luciferase activity.

Cell proliferation assay
Cells were seeded (1 × 103 cells per well) in 96-well plates and incubated for 24 hours and then treated with different doses of silybin. After incubation for 12, 24, 48, or 72 hours, 20 μL of CellTiter96 Aqueous One Solution (Promega) was added, and then cells were incubated for 1 hour at 37°C in a 5% CO2 incubator. Absorbance was measured at 492 nm. For anchorage-independent cell growth assessment, cells suspended in 10% Basal Medium Eagle medium were added to 0.3% agar with vehicle, 20, 40 or 80 μmol/L silybin in a top layer over a base layer of 0.5% agar with vehicle, 20, 40, or 80 μmol/L silybin. The cultures were maintained at 37°C in a 5% CO2 incubator for 3 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.6.1) program (Media Cybernetics).

In vitro and ex vivo pull-down assay
Recombinant human MEK1, MEK2, or RSK2 (200 ng) and SK-MEL-5 cell lysates (500 μg) were incubated with silybin-Sepharose 4B (or Sepharose 4B only as a control) beads (50 μL; 50% slurry) in reaction buffer (50 mMol/L Tris, pH 7.5, 5 mMol/L EDTA, 150 mMol/L NaCl, 1 mMol/L DTT, 0.01% NP-40, and 2 ng/mL bovine serum albumin).
After incubation with gentle rocking overnight at 4°C, the beads were washed 5 times with buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, and 0.01% NP-40), and binding was visualized by Western blotting.

Western blot analysis

Cells were disrupted on ice for 30 minutes in cell lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonylfluoride). After centrifugation at 20,817 × g for 15 minutes, the supernatant fraction was harvested as the total cellular protein extract. The protein concentration was determined using the Bio-Rad protein assay. The total cellular protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes in 20 mmol/L Tris-HCl (pH 8.0), containing 150 mmol/L glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in 1× TBS containing 0.05% Tween 20 (TBS-T) and incubated with antibodies against phosphorylated BRAF (pBRAF), Braf, MEK1/2, ERK1/2, pRSK2, RSK2, cyclin D1, or β-actin. Blots were washed 3 times in 1× TBS-T buffer, followed by the incubation with the appropriate horseradish peroxidase-linked immunoglobulin G. The specific proteins in the blots were visualized using the enhanced chemiluminescence detection reagent.

Xenograft mouse model

Female BALB/c (nu/nu) mice, 6 weeks old, were purchased from Charles River and were housed in a light/dark cycle of 12/12 hours and fed with rodent chow and water ad libitum. All animal experiments were approved by the Kyungpook National University Ethics Research Board (Dae-gu, South Korea). Mice were divided into 3 groups: (i) mice injected with SK-MEL-5 cells and treated with silybin, (ii) mice injected with SK-MEL-5 cells and treated with vehicle, and (iii) mice receiving no cells but treated with silybin to assess toxicity. SK-MEL-5 cells (3 × 10^6 in 100 μL PBS with 100 μL Matrigel) were injected subcutaneously into the right hind flank of 2 groups of mice (n = 10). Tumor volume (length × width × depth) was measured once a week. After 10 days of implantation, 2 groups (n = 10) were given silybin (dissolved in 5% dimethyl sulfoxide and 10% Tween-20 in PBS) intraperitoneally 3 times a week at a dose of 100 mg/kg body weight for 4 consecutive weeks. The third group received vehicle only. Body weights were recorded every week. Xenograft tumors were weighed and frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin.

Hematoxylin and eosin staining and immunohistochemistry

Tumor tissues from mice were embedded in paraffin blocks and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry. Tumor tissues were deparaffinized and hydrated, then permeabized with 0.5% Triton X-100/1× PBS for 10 minutes. Tissues were then hybridized with Ki-67 (1:50), pERK1/2 (1:200), pRSK2 (1:200) as the primary antibody, and biotinylated goat anti-rabbit or mouse IgG antibody was used as the secondary antibody. An ABC kit (Vector Laboratories, Inc.) was used to detect the protein targets according to the manufacturer’s instructions. After developing with 3,3′-diaminobenzidine, the sections were counterstained with hematoxylin (H&E). All sections were observed by microscope (×200 magnification) and the Image-Pro Plus software (v. 6.1) program (Media Cybernetics).

Statistical analysis

All quantitative results are expressed as mean value ± SD. Statistically significant differences were obtained using the Student t test or by one-way ANOVA. A P value of <0.05 was considered to be statistically significant.

Results

Silybin inhibits the proliferation of melanoma cells

Because silybin was identified as an inhibitor of BRAF-MEK1/2/ERK1/2 signaling through our virtual screening of a variety of FDA drug/supplementary/integrative medicine lead databases, we investigated the effect of silybin on melanoma cell growth. The BRAF, MEK1/2, ERK1/2, and RSK2 proteins were constitutively phosphorylated and highly expressed in SK-MEL-5 and SK-MEL-28 melanoma cells, whereas these proteins were barely activated in normal human dermal fibroblasts (NHDF; Supplementary Fig. S1). Incubation with silybin significantly reduced the viability of SK-MEL-5 and SK-MEL-28 melanoma cells in a concentration- and time-dependent manner, but had no effect on the viability of NHDFs (Fig. 1A). Only a very high concentration of silybin reduced the viability of SK-MEL-2 cells (Fig. 1A). Anchorage-independent cell growth assay results also showed that silybin significantly attenuated the growth of SK-MEL-5 and SK-MEL-28, but not SK-MEL-2, melanoma cells (Fig. 1B). Treatment with silybin altered the cell-cycle distribution in SK-MEL-5 cells by inducing cell-cycle arrest (Fig. 1C). Silybin showed G1 phase arrest in SK-MEL-28 cells at a concentration of 80 μmol/L only but did not exhibit significant cell-cycle arrest in SK-MEL-2 cells (Fig. 1C).

Silybin inhibits SK-MEL-5 melanoma tumor growth in a xenograft mouse model

The inhibitory effect of silybin on melanoma cell growth was confirmed in an in vivo xenograft mouse model. Intraperitoneal administration of silybin (100 mg/kg body weight) 3 times a week for 4 weeks significantly reduced the volume of SK-MEL-5 melanoma cell xenograft tumors in nude mice (Fig. 2A, left) without causing significant changes in animal body weight (Fig. 2A, right). Immunohistochemical analysis of xenograft tumors showed that the expression of the cell proliferation marker Ki-67 and phosphorylation of ERK1/2 and RSK2 were markedly decreased in silybin-
Silybin inhibits the proliferation of melanoma cells. A, human melanoma cells (SK-MEL-5, SK-MEL-28, and SK-MEL-2) were treated or not treated with varying concentrations of silybin for 12, 24, 48, or 72 hours. Cell proliferation was analyzed by the MTS assay. The asterisk (*) indicates a significant ($P < 0.01$) decrease in proliferation compared with untreated control. B, melanoma cells were incubated with silybin (0, 20, 40, or 80 μmol/L) and subjected to an anchorage-independent cell growth assay. Data are represented as mean ± SD of values from triplicate samples, and similar results were obtained from 2 independent experiments. The asterisks (*, $P < 0.05$, **, $P < 0.01$) indicate a significant decrease in colony formation in cells treated with silybin compared with untreated control. C, silybin induces G1 cell-cycle arrest in SK-MEL-5 and SK-MEL-28 cells, but not in SK-MEL-2 cells. Cells were starved for 24 hours and treated with silybin (0, 40, or 80 μmol/L) and then incubated for an additional 48 hours. Cell cycle was analyzed by staining with propidium iodide. The asterisk (*) indicates a significant difference ($P < 0.05$) between untreated control and treated cells.
treated tumors compared with vehicle-treated tumors (Fig. 2B).

**Silybin directly binds to and inhibits the kinase activity of MEK1/2 and RSK2**

We determined whether silybin interferes with the components of the BRAF/MEK/ERK/RSK2 signaling pathway in SK-MEL-5 and SK-MEL-28 melanoma cells. Treatment with silybin inhibited the constitutive phosphorylation of ERK1/2 and RSK2 (Fig. 3A) with more pronounced effects on SK-MEL-5 cells compared with its effects on SK-MEL-28 cells. It might be due to the higher expression of phosphorylated MEK1/2 in SK-MEL-5 cells. Silybin did not affect the phosphorylation of BRAF or MEK1/2 (Fig. 3A). Because ERK1/2 is a substrate of MEK1/2, which is regulated by upstream BRAF, we used an in vitro kinase assay to evaluate the effect of silybin on the catalytic activities of these kinases. While silybin inhibited BRAF activity only at a high concentration (80 μmol/L; Fig. 3B, a), the compound attenuated the kinase activity of MEK1 (Fig. 3B, b), MEK2 (Fig. 3B, c), and RSK2 (Fig. 3B, d) in a concentration-dependent manner. Although silybin diminished the phosphorylation of ERK1/2 in SK-MEL-5 cells, it failed to inhibit ERK2 activity in an in vitro kinase assay (Fig. 3B-e). To determine whether silybin can interact with these kinases, we conducted in vitro (Fig. 3C, a–c) and ex vivo (Fig. 3C, d and e) pull-down assays. Silybin was shown to interact with recombinant active MEK1, MEK2, and RSK2 proteins (Fig. 3C, a–c). The interaction between silybin and MEK1/2 or RSK2 was further confirmed by pull-down assay using SK-MEL-5 cell lysates (Fig. 3C, d and e). To determine whether the binding of silybin to MEK1/2 or RSK2 occurs in an ATP-competitive manner, we conducted an ATP (1, 10, or 100 μmol/L) competitive kinase pull-down assay. Results showed that silybin bound to MEK1, MEK2, and RSK2 in an ATP-competitive manner (Fig. 3D, a–c). This result suggests that...
Silybin inhibits the BRAF/MEK/ERK signaling pathway by directly binding to MEK1/2 and RSK to suppress their activities.

**Confirmation of the interaction between silybin and MEK1/2 or RSK2 using a computational docking study**

Silybin is a flavonoid containing polyphenolic groups (Fig. 4A). The hierarchical docking algorithm, Glide (22, 23), from Schrödinger-Maestro v9.2 Suite (25), was used for docking experiments to assess the possible binding modes between MEK1 or RSK2 and silybin. To capture the ligand-induced conformational changes in enzyme active sites, we conducted flexible-ligand flexible-protein docking using the IFD Module (24). The computational model of the silybin-MEK1 complex is shown in Fig. 4B (top). Silybin forms 4 hydrogen bonds with the protein residues in the ATP-binding pocket (Fig. 4B, top right): 2 with the backbone atoms of the hinge residues of Glu144 and Met146 and the other 2 with the side chain atoms of Lys97 and Asp208, respectively. The computational models of the silybin–RSK2 complex are shown in Fig. 4B (bottom). The docking of silybin to RSK2 from IFD simulations suggested that silybin could bind inside the ATP-binding pocket of either the N-terminal or C-terminal kinase domain. For the latter, silybin forms 5 hydrogen bonds with RSK2 (Fig. 4B, bottom right): 1 involved in the backbone atom of the hinge residue Met496 and the other 4 formed with the side-chain atoms of Thr493, Thr500, Thr501, and Thr502.
Asn544, Lys541, and Lys451, respectively. These computational modeling results indicate that silybin exerted ATP-competitive inhibitory effects against MEK1 or RSK2.

Silybin suppresses the expression of cyclin D1 by inhibiting the transcriptional activity of NF-κB, AP-1, and STAT3

Because MEK1/2 and RSK2 are known to transmit signals downstream to a variety of transcription factors, which regulate the transcriptional activity of proteins involved in the cell proliferation, we determined whether the inhibitory effect of silybin on the activity of these kinases could affect the transcriptional activity of NF-κB, AP-1, or STAT3. Because MEK1/2 is an upstream kinase of RSK2, we knocked down MEK1/2 (sh-MEK1/2) in SK-MEL-5 cells, and found that silencing MEK1/2 reduced the proliferation of SK-MEL-5 melanoma cells (Supplementary Fig. S2A). The inhibitory effect of silybin on the viability of SK-MEL-5 cells was abrogated when the cells were transfected with sh-MEK1/2 as compared with cells transfected with sh-Mock (Supplementary Fig. S2B). Moreover, knockdown of MEK1/2 attenuated the constitutive transcriptional activity of NF-κB, AP-1, or STAT3 in SK-MEL-5 cells (Supplementary Fig. S2C). We conducted luciferase reporter gene assays in silybin-treated SK-MEL-5 or SK-MEL-28-cells transfected with NF-κB-, AP-1-, or STAT3-luciferase constructs, and found that silybin significantly inhibited the transcriptional activity of NF-κB, AP-1, and STAT3 (Fig. 5A, a–f). A common target gene of these transcription factors is cyclin D1, which is a cell-cycle regulatory protein. Western blot analysis results indicated that treatment with silybin attenuated the expression of cyclin D1 in SK-MEL-5 and SK-MEL-28 melanoma cells (Fig. 5B).

Discussion

Aberrant activation of BRAF signaling has been implicated in the pathogenesis of melanoma (26–28), a deadly form of skin cancer that afflicts millions of people throughout the world. The modulation of BRAF signaling would be a rational approach for the prevention and therapy of melanoma. To identify an inhibitor that can act on multiple kinases in the BRAF-signaling cascade, we screened the FDA drug database to search for a drug molecule that can target kinases of this oncogenic signal transduction pathway. During ligand–protein docking in virtual screening, each kinase target was treated as a solid object but the drug compound was fully flexible. Each screening process targeted 1 of the 3 kinases, BRAF, MEK1/2, or ERK1/2.
2, of the BRAF pathway and provided a docking scoring of the tested drug molecules and their relative rank in binding affinity with the selected kinase targets. After checking the first 50 drug leads for each kinase target, we found that silybin was ranked in consensus inside this range. Supplementary Table S1 shows the binding energy of silybin to these 3 kinases and its relative rank among all the drugs screened from the FDA drug database. These docking results indicated that silybin could possibly serve as a "multiplex kinase inhibitor" of the BRAF pathway. Here, we provide the novel finding that silybin directly interacts with MEK1/2 and RSK2, and inhibits their catalytic activity, thereby suppressing the growth of SK-MEL-5 or SK-MEL-28 melanoma cells.

In a previous study, Nakashima and colleagues (30) reported that silybin A and B, 2 isoforms of silybin, attenuated proliferation of mouse melanoma B16 4A5 cells in culture. In agreement with the later study, our findings indicated that silybin decreased the viability of SK-MEL-2, SK-MEL-5, and SK-MEL-28 cells and reduced the growth of SK-MEL-5 human melanoma cells as a xenograft in nude mice and supports the potential of silybin to prevent or treat melanomas. The results of the in vitro kinase assay showing that silybin significantly inhibited the catalytic activity of MEK1 and MEK2 confirmed our virtual screening results, which predicted MEK1/2 as a molecular target of silybin to suppress melanoma cell growth. Moreover, the in vitro kinase assay results revealed that silybin attenuated the kinase activity of RSK2, which is a downstream kinase of ERK2. Although silybin did not affect the activity of ERK2 in the in vitro kinase assay, incubation of SK-MEL-5 cells with silybin reduced the phosphorylation of ERK1/2 as well as that of RSK2, suggesting that RSK2 is another molecular target of silybin. To confirm that silybin targets MEK1/2 and RSK2, we conducted a binding assay using Sepharose 4B beads conjugated with silybin in the presence of active MEK1, MEK2, or RSK2 in both cultured cells and in vitro. Silybin interacted with MEK1/2 and RSK2 in an ATP-competitive manner. Further analysis of docking studies by computer modeling showed that silybin interacted at the ATP-binding pocket of MEK1/2 and RSK2, confirming that these kinases are bona fide targets of silybin in suppressing melanoma cell growth.

Although MEK1/2 and RSK2 have been identified as direct targets of silybin, we extended our study to determine whether silybin inhibited the activity of other kinases, such as...
as Akt, p38 mitogen-activated protein kinase, and c-Jun-N-terminal kinases, which have also been reported to play roles in melanoma cell proliferation. Silybin had no effect on the activity of any of these kinases (data not shown).

Because silybin suppressed the proliferation of BRAF-mutated cell lines, SK-MEL-5 and SK-MEL-28, more than the NRAS-mutated SK-MEL-2 cells, we suggest that silybin targets the constitutive BRAF signaling pathway, but not the NRAS signaling pathway to block melanoma cell proliferation.

Furthermore, the activation of kinases of the BRAF-signaling cascade turns on the transcriptional activity of downstream transcription factors, such as NF-κB, AP-1, and STAT3 (31–33). The direct binding and subsequent blockade of MEK1/2 and RSK2 by silybin resulted in a significant inhibition of the transcriptional activity of NF-κB, AP-1, and STAT3 in silybin-treated SK-MEL-5 and SK-MEL-28 cells. A common target gene of these transcription factors is the cell-cycle regulatory protein, cyclin D1, which was markedly downregulated after treatment of cells with silybin.

Figure 5. Silybin inhibits the transcriptional activity of NF-κB, AP-1, and STAT3. A, SK-MEL-5 or SK-MEL-28 cells were transiently transfected with the luciferase reporter gene constructs of NF-κB, AP-1, or STAT3 and incubated with silybin (0, 40, or 80 μmol/L). Luciferase activity was measured as described in Materials and Methods. Silybin inhibited (a and d) NF-κB, (b and e) AP-1, and (c and f) STAT3 transcriptional activity in SK-MEL-5 (a–c) or SK-MEL-28 (d–f) cells. Data are shown as mean ± SD of values from triplicate samples and similar results were obtained from 3 independent experiments. The asterisks (*, P < 0.05, **, P < 0.01) indicate a significant decrease in relative luciferase activity. B, effect of silybin on the expression of cyclin D1 in SK-MEL-5 and SK-MEL-28 cells.
inhibitory effect of silybin on the expression of cyclin D1 helps to explain the effect of the compound on the cell-cycle arrest observed in SK-MEL-5 and SK-MEL-28 cells. In conclusion, silybin attenuated melanoma cell growth by modulating the activity of multiple kinases (MEK1/2, ERK2, RSK2) of the BRAF signaling pathway, thereby blocking the activity of NF-κB, AP-1, and STAT3, and inducing cell-cycle arrest at the G1 phase through downregulation of cyclin D1 (Fig. 6).

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

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References


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