Gossypin as a Novel Selective Dual Inhibitor of v-raf Murine Sarcoma Viral Oncogene Homolog B1 and Cyclin-Dependent Kinase 4 for Melanoma

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Abstract

Mutation in the BRAF gene (BRAFV600E) exists in nearly 70% of human melanomas. Targeted therapy against BRAFV600E kinase using a recently identified RAF-selective inhibitor, PLX4032, has been successful in early clinical trials. However, in patients with the normal BRAF allele (wild-type), PLX4032 is protumorigenic. This conundrum identifies the unmet need for novel therapeutic agents to target BRAFV600E kinase that are not counterproductive. We have identified gossypin, a pentahydroxy flavone, as a potent antimelanoma agent. Gossypin inhibited human melanoma cell proliferation, in vitro, in melanoma cell lines that harbor both BRAFV600E kinase and cyclin-dependent kinase 4 (CDK4) as well as in cells with BRAF wild-type allele. Gossypin inhibited kinase activities of BRAFV600E and CDK4, in vitro, possibly through direct binding of gossypin with these kinases, as confirmed by molecular docking studies. For cells harboring the BRAFV600E, gossypin inhibited cell proliferation through abrogation of the MEK–ERK–cyclin D1 pathway and in cells with BRAF wild-type allele, through attenuation of the retinoblastoma–cyclin D1 pathway. Furthermore, gossypin significantly inhibited melanoma growth in an organotypic three-dimensional skin culture mimicking human skin. Gossypin (10 and 100 mg/kg) treatment for 10 days in human melanoma (A375) cell xenograft tumors harboring BRAFV600E significantly reduced tumor volume through induction of apoptosis and increased survival rate in mice, and the effect was significantly superior to that of PLX4032 (10 mg/kg) or roscovitine 10 mg/kg. In summary, this study identified gossypin as a novel agent with dual inhibitory effects for BRAFV600E kinase and CDK4 for treatment of melanoma. Mol Cancer Ther; 12(4); 361–72. ©2013 AACR.

Introduction

Melanoma is the most aggressive and metastatic form of skin cancer, generated from transformed melanocytes that are originally derived from the embryonic neural crest. In the United States, incidence of melanoma has doubled in the last 30 decades (1). According to the NIH, approximately 68,130 new cases and 8,700 deaths were reported in 2010 alone. Currently, therapies approved for melanoma by the U.S. Food and Drug Administration (FDA) are high dose of interleukin (IL)-2 and dacarbazine (2). However, both these agents are associated with very low response rates and neither of them improves overall survival (3, 4).

Among the genes that have been associated with melanoma development, the serine/threonine kinase BRAF and cyclin-dependent kinases (CDK) are very important. Of all, 60% to 70% melanomas harbor BRAF missense mutations that result in the substitution of glutamic acid for valine at amino acid 600 (BRAFV600E; ref. 5). This causes destabilization of the inactive kinase conformation by switching the equilibrium toward active form rendering BRAFV600E 500 times more active than wild-type BRAF (6, 7). The constitutively active BRAFV600E can stimulate MEK–ERK pathway in cancer cells, and hence targeted inhibition of BRAFV600E is ideal for the treatment of melanoma. Recently, a small-molecule inhibitor for BRAFV600E, PLX4032 (Fig. 1A) showed promising results in patients with melanoma harboring BRAFV600E mutation (8, 9). However, tumors with RAS mutation were not sensitive to PLX4032 and, paradoxically, PLX4032 activated MEK–ERK pathway in BRAF wild-type cells (10). CDKs are strongly involved in regulating the progression of the cell cycle and levels of CDKs are upregulated in melanoma (11, 12). The most common aberration to cell-cycle control in melanoma affects the CDKN2A gene. Approximately, 50% of melanomas have CDKN2A locus mutations, leading to loss of function of the downstream target, p16INK4A, an inhibitor of CDK4
This can be either due to deletion of the CDKN2A gene or epigenetic modifications of the CDKN2A gene promoter leading to loss of negative regulation of the cell cycle, causing increased proliferation (14). Therefore, in addition to BRAFV600E, CDKs are yet another promising target for melanoma therapy.

Gossypin (3,5,8,3',4'-pentahydroxy-7-O-glucosyl flavone) is a natural compound isolated from Hibiscus vitifolius, which possesses anti-inflammatory properties (refs. 15, 16; Fig. 1A). Extracts from Hibiscus vitifolius are traditionally used for the treatment of diseases such as diabetes, jaundice, and inflammation (17, 18). Other
effects of gossypin are its ability to inhibit galactose-induced cataract formation (15), reduction of β-amyloid-induced toxicity (19), protection against carbon tetrachloride–induced toxicity and bis(2-chloroethyl)sulfide–induced dermal toxicity (20, 21), and apoptosis in myeloma cells (22). In the present study, we have tested the effect of gossypin on melanoma cell growth both in vitro and in vivo. Gossypin inhibited G1-S-phase cell cycle transition in human melanoma cells harboring BRAFV600E and CDK4 mutations through a direct interaction with these kinases, in vitro. We further showed that inhibition of cell proliferation by gossypin in melanoma cells with BRAFV600E mutation is through inhibition of MEK–ERK–cyclin D1 pathway, whereas retinoblastoma (Rb)–cyclin D1 pathway was inhibited in melanoma cells with wild-type BRAF allele. We also found that treatment with cold 70% ethanol, washed with cold PBS, and stained with propidium iodide (PI; 50 μg/mL in PBS) followed by the addition of ribonuclease (10 μg/mL) and incubation for 4 hours in the dark. DNA content was analyzed by flow cytometry [Becton Dickinson FACScan Flow Cytometer (BD Biosciences)] and analyzed with FACS Express software (De Novo Software).

Materials and Methods

Cell culture

A375, WM1552C, WM793B, SKMEL-31, and SKMEL-28 melanoma cell lines were purchased from American Type Culture Collection and 1205Lu cell line was procured from Coriell Institute for Biomedical Research (Camden, NJ). 1205Lu cells were originally generated from human metastatic melanoma 973B cells xenografted on nude mice (23). Karyotype analysis of 1205Lu cells revealed that these cells contain mouse chromosomes (80%) in addition to human chromosomes (20%; unpublished data). Because these cells contain both BRAFV600E and CDK4 mutations and are aggressively metastatic in nature, we have used 1205Lu cells in our experiments despite their anomalous karyotype. All the cell lines were maintained in proper culture conditions as per provider’s instructions. The cell lines were authenticated by comparing the tumor mutation profile determined with published reports.

Cell proliferation assay

Cytotoxicity of gossypin was assessed using Cell Titre Glo assay (Promega) as per manufacturer’s instructions. Briefly, 5 × 10^4 cells were seeded in 96-well plates and incubated with gossypin (1–200 μmol/L) or dimethyl sulfoxide (DMSO; 0.02% v/v) for 24, 48, and 72 hours at 37°C and cell viability was measured using a Fluoroscan plate reader (Thermo; ref. 24).

Soft agar colony formation assay

Basal layer of agar was prepared by mixing 1% DNA grade agar melted and cooled to 40°C with an equal volume of (2×) Dulbecco’s Modified Eagle’s Medium (DMEM) to obtain 0.5% agar that was dispersed in a well plate and allowed to solidify. A total of 0.6% agar was prepared in RPMI medium and mixed together with A375 cells (0.5 × 10^6 cells/mL) and immediately plated on the basal layer in the presence or absence of gossypin. The cultures were incubated at 37°C in a CO2 incubator for 2 weeks, and colonies were stained with 0.005% crystal violet and observed under a light microscope.

Flow cytometry

In T25 flasks, 0.05 × 10^6 cells were seeded and treated after 24 hours with different concentrations of gossypin for 48 hours. The cells were harvested by trypsinization, pelleted at 1,200 rpm, and washed twice with cold PBS. The cells were fixed with cold PBS, and stained with propidium iodide (PI; 50 μg/mL in PBS) followed by the addition of ribonuclease (10 μg/mL) and incubation for 4 hours in the dark. DNA content was analyzed by flow cytometry and western blot analysis.

Western blot analysis

Cells were seeded in 10-cm dishes at 70% confluence 1 day before the treatment. Different concentrations of gossypin (10–100 μmol/L) were added and incubated for 48 hours in a CO2 incubator at 37°C. Cells were harvested and immediately lysed with radioimmunoprecipitation assay buffer (RIPA) buffer containing protease inhibitors (Sigma), and centrifuged for 15 minutes at 10,000 × g. An equal quantity of protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) and was blocked for 1 hour with 5% nonfat dry milk followed by incubation with primary and secondary antibodies. The antibodies against the following proteins were used: β-actin, cyclin D1, S100 (Santa Cruz Biotechnologies), phospho-extracellular signal-regulated kinase (ERK; Thr 202/204), phospho-mitogen-activated protein/ERK (MEK; Ser221), phospho-Rb (Ser 780), total ERK and MEK (Cell signaling Technology) p27 (BD Biosciences), and Ki67 (Abcam). The chemiluminescence signal was developed with ECL-plus reagent (Bio-Rad) and detected by autoradiography.

Caspase-3/7 assay

Caspase-3/7 activity in tumor tissues was measured using Caspase-Glo assay kit (Promega), as described before (25). Briefly, the tumor tissues were homogenized in homogenization buffer (25 mmol/L HEPES, pH 7.5, 5 mmol/L MgCl2, and 1 mmol/L EGTA), protease inhibitors, and the homogenate was centrifuged at 13,000 rpm at 4°C for 15 minutes. To 10 μL of the supernatant containing protein was added to an equal volume of the assay reagent and incubated at room temperature for 2 hours. The luminescence was measured using a luminometer.

In vitro CDK4 kinase assay

Glutathione S-transferase (GST)-CDK4 and GST-tagged cyclin D1 as cyclin–CDK complex were purchased.
from Cell Signaling Technology. We used this enzyme complex to phosphorylate Rb as a substrate for CDK4. Five microgram of Rb (Roche) was incubated with CDK4–cyclin D1 complex for 30 minutes at 30°C in 30 μL reactions containing 60 mmol/L HEPES pH 7.4, 3 mmol/L MgCl2, 3 mmol/L MnCl2, 1.2 mmol/L dithiothreitol, 20 μmol/L ATP, and 5 μCi [32P] ATP and varying concentrations of gossypin were tested for their effect on CDK4 kinase activity. The kinase reaction was stopped by adding 4× SDS dye, followed by 12% SDS-PAGE to resolve the phospho-Rb and enzyme complex. The separated proteins were transferred onto a nitrocellulose membrane and quantified using autoradiography.

**In vitro BRAFV600E kinase assay**

BRAFV600E kinase assay was conducted by Z'-LYTE biochemical assay using Select Screen Biochemical Kinase Profiling Service (Invitrogen). The assay uses a fluorescence-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and nonphosphorylated peptides to proteolytic cleavage.

**Molecular docking and dynamics studies of gossypin**

Binding mode of gossypin with BRAFV600E kinase and CDK4 was analyzed by flexible docking simulations. The crystal structure of BRAFV600E oncogenic mutant complex with PLX4032 (PDB ID: 3OG7) and CDK4 complexes with D-type cyclin (PDB ID: 2W96) was selected as receptors for the docking studies (26, 27). The missing residues in the crystal structures were modeled and 1,000 steps of each conjugate gradient and steepest descent energy minimization was applied with a force filed OPLS-2001 using Schrödinger Maestro 9.1 program (Schrödinger, LLC; refs. 28, 29). The dielectric constant and van der Waals cutoff was set as 78 and 12 Å, respectively. Proteins’ ligand structures were further prepared for docking using the protein preparation wizard and the ligprep modules using OPLS-2001 and Merck molecular force field (MMFF) (30), respectively. 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**Real-time quantitative RT-PCR**

RNA was extracted from cells using a TRIzol (Sigma)/chloroform method and cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega) and an oligo(dT)15 primer. Quantitative real time reverse transcription PCR (RT-PCR) was conducted in triplicate using an ABI prism real-time PCR (MJ Research) along-side no-template controls. Each 25 μL reaction included 12.5 μL DyNamo master mix (Finnzymes) and 10 pmol of primers. The relative levels of p21, p27, cyclin D1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were calculated using the ACt method (31). The primers used were the following:

GAPDH: 5'-GGCTTCCAAAAAGGATAGACC-3' (forward) 5'-AGGGTCTACATGGAATGCG-3' (reverse) Cyclin D1: 5'-ACGAAGGTCTCGCCGTGTT-3' (forward) 5'-CCGCTGGCCATGAATCTACCT-3' (reverse) p27 (Kip) 5'-TGCAACCGAGTTCTCTACTCAA-3' (forward) 5'-CAAGCAGTGTATTGATGATAACAGGA-3' (reverse).

**In vitro invasion assay**

Invasion assay was conducted using tissue culture–treated Transwell chamber with 8-μm pore membranes. The base of the chamber was filled with 0.8 mL serum-reduced medium as chemoattractant. A375 cells (0.02 × 10⁶) were labeled with CellTracker Green (Molecular Probes-Invitrogen) and plated on top of growth factor–reduced Matrigel (BD Biosciences) in the presence of gossypin or 0.2% DMSO. The invasion chambers were incubated at 37°C in 5% CO₂ for 24 hours. The cells that did not invade through the Matrigel, were manually removed with cotton tip applicators. The invaded cells were visualized on the bottom of the coated membranes with a fluorescence microscope at ×20 objective (32). The relative fluorescence was quantified using Biorad-GS800 calibrated imaging densitometer installed with Quantity One software (Bio-Rad).

**Three-dimensional skin reconstruction assay**

Three-dimensional skin reconstruction (3-DSR) models of A375 melanoma cells were prepared by plating single-cell suspensions of normal human epidermal keratinocytes and A375 melanoma cells at a 1:10 ratio on fibroblast-contracted collagen gels within cell culture inserts. They were allowed to grow and differentiate in DMEM-based serum-free medium, forming three-dimensional (3D), highly differentiated, full-thickness, skin such as tissues. These 3-DSR models were purchased from MatTek. Eight-day-old culture inserts were incubated in duplicate with serum-free medium containing either DMSO or gossypin. The medium was replenished every other day and cell cultures were collected after 2 or 7 days and fixed with 10% formalin. For immunohistochemistry, culture inserts were paraffin embedded, serial sectioned, and analyzed by hematoxylin and eosin (H&E) staining.

**Immunohistochemistry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

The paraffin-embedded 3D tissue construct or tumor tissues sections were blocked with 10% goat serum in PBS for 20 minutes and incubated with anti-S100 or anti-Ki67.
antibody, phospho-MEK, and phospho-ERK in blocking buffer for 2 hours. The slides were washed with PBS containing 0.1% Triton X-100 (wash buffer), incubated with secondary antibody [goat-anti-mouse immunoglobulin G (IgG); Promega Corporation] conjugated with alkaline phosphatase in blocking buffer for 1 hour, washed with washing buffer, and mounted. Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, in situ, as described by the manufacturer (BioVision Technologies). Briefly, the tissue constructs were fixed in 4% paraformaldehyde solution, embedded, and sectioned. After the sections were permeabilized, double-stranded breaks were labeled with biotinylated nucleotide and terminal deoxynucleotidyl transferase enzyme. After the reactions were terminated, apoptosis was indicated by the dark brown color of horseradish peroxidase–labeled streptavidin bound to the biotinylated nucleotides. Three independent TUNEL assays were conducted with DNase-I–treated tissue sections as positive controls and negative controls without the enzyme.

**Tumor xenograft studies**

All experiments were carried out in compliance with the principles of Guide for the Care and Use of Laboratory Animals. All procedures were approved before use by Institutional Animal Care and Use Committee of Texas Biomedical Research Institute (San Antonio, TX). Uniform suspensions of human melanoma A375 cells (1 × 10⁶) in 100 µL PBS were injected subcutaneously into the right flank of 4- to 5-week-old female athymic nude mice (Harlan Industries). After 10 days, when the tumor diameter reached 10 mm, the mice were randomly allocated to 5 groups of each containing 10 animals. Group 1 served as the untreated control, groups 2 and 3 received gossypin at 10 mg and 100 mg/kg, respectively, group 4 received PLX4032 (10 mg/kg), and group 5 was administered with roscovitine (10 mg/kg) orally. All drugs were suspended in PBS followed by sonication (15 seconds with an intermittent interval of 5 seconds) for 1 minute. Tumors were allowed to reach palpability before drug intervention. Tumor size was measured every 5 days using vernier calipers and tumor volume was calculated using the ellipsoid formula: 

\[
V = \frac{4}{3} \pi \left(\frac{D \times d}{2}\right)², \quad \text{where } D \text{ is the large diameter of the tumor and } d \text{ represents the small diameter.}
\]

On day 31, the mice were euthanized and tumors were harvested.

**Statistical methods**

Statistical analysis was conducted by the Student t test. Differences between the groups were considered significant when the P value was 0.05 or less and survival analysis was conducted with Kaplan–Meier plot.

**Results**

**Gossypin exhibits cytotoxicity toward human melanoma cell lines**

To study the in vitro cytotoxicity effects of gossypin on melanoma cells, we have used the following human melanoma cell lines: 1205Lu, SKML-28 (BRAFV600E kinase and CDK4), SKMEL-31 (BRAF wild-type kinase), A375 (BRAFV600E kinase), WM793B, and WM1552C represent vertical and radial growth phase of melanoma with BRAFV600E kinase mutation. All the cell lines were treated with various concentrations (1–200 µmol/L) of gossypin for 24 to 72 hours. Cytotoxic effect of gossypin was dose-dependent in all the tested cell lines, irrespective of their mutation status. While gossypin-mediated cytotoxic effect of A375, WM793B, and WM1552C cells were time-dependent, such an effect was absent in 1205Lu and SKML 31 cells. However, gossypin treatment exhibited cytostatic effect in SKMEL-28 cells (Fig. 1B–G). Growth inhibitory effect of gossypin on melanoma cell proliferation was further confirmed by colony formation assay using A375 cell line. Gossypin dose-dependently inhibited colony formation of A375 cells, compared with vehicle-treated controls, indicating that gossypin could prevent anchor-independent growth of melanoma cells (Fig. 1H).

**Gossypin inhibits invasion of A375 cells in vitro and in artificial human 3D skin culture model**

Effect of gossypin on melanoma cell invasion was tested in A375 cells using Transwell chamber invasion assay. Invasion and motility of A375 cells were significantly reduced by gossypin in a dose-dependent manner (Fig. 2A). While 25 µmol/L of gossypin caused a 50% inhibition of invasion, 50 µmol/L gossypin exhibited 90% of cellular invasion (Fig. 2B). This result shows the potency of gossypin in inhibiting cellular invasion, a key event in the process of melanoma metastasis.

Further we have tested the effect of gossypin on inhibiting cellular invasion in an organotypic skin culture system using a full-thickness 3-DSR model of A375 melanoma cells (Fig. 2C). The skin cultures were treated with 2 different concentrations of gossypin (25–50 µmol/L), either for 2 or 7 days and cultures treated with DMSO was as control. H&E staining indicated that DMSO-treated cultures developed massive invasion of melanoma cells by day 7, whereas gossypin treatment inhibited proliferation. DMSO-treated cultures developed massive invasion of melanoma cells by day 7, whereas the gossypin-treated cultures showed less infiltration and less proliferation of cells, compared with DMSO control (Fig. 2C). Immunohistochemical analysis of S100 (marker for melanoma) and Ki67 (marker for proliferation) revealed that the DMSO-treated cultures have more S100 and Ki67-positive cells than the gossypin-treated cultures (Fig. 2D), suggesting that gossypin could potentially inhibit proliferation of melanoma cells. The TUNEL assay of the skin cultures revealed that prolonged (7 day) treatment with gossypin could induce apoptosis in melanoma cells (Fig. 2D). These findings indicate that gossypin could inhibit both proliferation and invasion of melanoma cells through induction of apoptosis.

**Gossypin inhibits G1–S transition in melanoma cells**

To determine the mode of action of gossypin on melanoma cells, A375 cells were treated either with DMSO or
varying concentrations of gossypin (25, 50, and 100 µmol/L) for 48 hours and subjected to flow cytometry analysis. Gossypin treatment resulted in the accumulation of cells in the G1-phase, whereas the number of cells in S-phase was decreased, suggesting that gossypin attenuates melanoma cell growth through inhibition of G1–S phase transition (Fig. 3A–D). Because CDK4 kinase has been reported to mediate G1–S phase transition (33), gossypin’s ability to inhibit CDK4 activity, in vitro, was tested. Gossypin dose-dependently inhibited CDK4 kinase activity as judged from the decreased phosphorylation of Rb suggesting that inhibition of G1–S phase transition by gossypin in melanoma cells was due to inhibition of CDK4 kinase activity (Fig. 3E).

Gossypin inhibits BRAF–MEK–ERK signaling in melanoma cells with BRAFV600E mutation

Expression of cyclin D1, phosphorylation of Rb, and G1–S cell-cycle progression are dependent on MEK–ERK signaling in tumors that contain BRAF mutations (34). To see whether gossypin has any direct effect on BRAF kinase activity, an in vitro kinase assay for BRAF wild-type and BRAFV600E in the presence or absence of gossypin was conducted. Gossypin inhibited kinase activity of both BRAF wild-type and BRAFV600E, however, highest inhibitory effect was observed toward BRAFV600E (74% for BRAFV600E and 30% for BRAF wild-type; Fig. 4A). Thus, gossypin has a preference for BRAFV600E, not BRAF wild-type kinase. Because inhibition of BRAF kinase activity will affect downstream signaling events, effect of gossypin on MEK–ERK pathway, the direct downstream target of BRAF kinase was assessed. We used 3 different kinds of cell lines for this study: SKMEL-31 cells with BRAF wild-type kinase, A375 cells with BRAFV600E, and 1205Lu cells with both BRAFV600E kinase and CDK4. While gossypin did not exert any inhibitory effect on MEK–ERK pathway in SKMEL-31 cells, MEK–ERK pathway was strongly inhibited by gossypin in both A375 cells as well as 1205Lu cells (Fig. 4B). However, at a higher concentration (100 µmol/L), gossypin inhibited MEK phosphorylation in SKMEL-31 cells without affecting ERK phosphorylation (Fig. 4B). These results further confirm our previous finding in Fig. 4A that gossypin preferentially binds BRAFV600E, not BRAF wild-type kinase.

Rb tumor suppressor protein, cyclin D1 and p27 are downstream effectors of MEK–ERK pathway and, furthermore, Rb protein is a direct target of CDK4/cyclin D1. Gossypin treatment decreased phosphorylation of Rb and expression of cyclin D1, dose-dependently, in all the 3 cell lines regardless BRAF kinase mutation status (Fig. 4C). Effect of gossypin on p27 expression was also tested, as downregulation of p27 due to MEK–ERK activities in melanoma cells have been reported (35). Levels of p27 were unaffected by gossypin treatment in SKMEL31 cells, whereas its expression was dose-dependently upregulated in A375 cells as well as 1205Lu cells (Fig. 4C). In A375 cells, mRNA levels of cyclin D1 were decreased, whereas p27 mRNA levels were increased by gossypin treatment (Fig. 4D).
Targeted Inhibition of Melanoma by Gossypin

Gossypin actively binds to CDK4 and BRAFV600E kinases in docking studies

From the docking studies, it was observed that the binding of gossypin in the active site of BRAFV600E is in such a manner that the flavonol skeleton was pierced into the active site and the glucosyl moiety was pointed toward the surface. This conformation helps gossypin to make various hydrogen bonds with the following residues such as D594, T529, Q530, C532, I463, and N580. The catechol moiety of gossypin forms hydrogen bonds with D594 and T529. It was observed that the hydroxyl groups at C3, C5, and C7 positions were involved in hydrogen bonding with the residues Q530, C532, and I463, respectively. Residues C532 and I463 also forms hydrogen bond with keto group of chromen moiety and hydroxyl group of glucosyl moiety, respectively. The residue N580 was found to interact through its O, OD1, and ND2 atoms with different hydroxyl groups of glucosyl moiety. There are 79 nonpolar contacts (≤4 Å) between BRAF and gossypin. The binding affinity obtained was −13.60 kcal/mol. The binding mode of gossypin in the active site of BRAF is shown in Fig. 5A.

The binding affinity of gossypin toward CDK4 was −10.21 kcal/mol. Out of 9 hydroxyl groups in gossypin, 7 were involved in hydrogen bonding with residues such as I12, V14, A16, R101, K142, and D158. The catechol moiety interacts with the protein residues K142 and A16 and hydroxyl group at C3 position of gossypin to form hydrogen bonds with D158. The keto group of the chromone moiety makes a hydrogen bond with K35. The glucoside moiety was arranged toward the solvent and was involved in the hydrogen bonds with I12, V14, and R101. Apart from these hydrogen bonds, 90 van der Waals contacts were also found to stabilize the protein ligand interactions. The flavonol skeleton of gossypin adopts a planar conformation and fits well in the active site cleft. The mode of binding gossypin at the active site of CDK4 is shown in Fig. 5B.

Gossypin maintain various hydrophobic interactions with nonpolar residues of BRAF and CDK4. In both interactions, the flavonol skeleton fit well in to the active site clefts, almost in a planar manner. At the same time, the glucosyl moiety points toward the surface. The binding affinity of some known inhibitors of BRAFV600E and CDK4 was also identified from the docking studies. It was observed that PLX4032 (a known inhibitor of BRAFV600E) is bound at the active site with a score of −15.55 kcal/mol at the same time 3FP and FAL (known inhibitors of CDK4) bound with a score of −6.10 and −6.28 kcal/mol, respectively.

Gossypin reduces tumor growth in nude mice bearing human melanoma xenografts

A375 human melanoma xenograft model was used to determine the effect of gossypin on tumor growth inhibition and overall survival. This study imitates the clinical...
scenario of patients where melanoma harbor BRAFV600E mutation. In addition, it also addresses the translational potential of our in vitro studies. Effects of gossypin (10 or 100 mg/kg) were compared with clinical candidate drugs, such as PLX4032 (10 mg/kg) and roscovitine (10 mg/kg), as described in Materials and Methods. Control animals administered with DMSO were euthanized because of excess tumor burden in the third week of treatment. Thus, significance in tumor reduction was calculated on the basis of the tumor volume at the third week with controls and at the fourth week with the other groups. In general, the overall survival rate of tumor-bearing animals were increased in the groups treated with gossypin when compared with those treated with PLX4032 or roscovitine (Fig. 6A). A significant and dose-dependent reduction in tumor volume was observed with gossypin treatment (10
Targeted Inhibition of Melanoma by Gossypin

Discussion

In the past few decades, melanoma research has made only limited progress and the median survival for patients with metastatic melanoma remains less than 10 months. Currently, dacarbazine is the only cytotoxic drug approved by the FDA for metastatic melanoma. However, dacarbazine response rates of 5% to 10% are connected with little survival improvement and serious hepatic side effects (36). Immunotherapy is another option for the treatment of melanoma. IL-2, the first cytokine permitted for use in patients with metastatic melanoma, has elicited sustained responses in a small subgroup of patients, providing hope that immunotherapy might be useful for this disease (3). Ipilimumab, a monoclonal antibody to CTLA-4, also has recently been approved by the FDA (37).

The discovery of the BRAF mutation, following a genome-wide analysis, revealed that 80% of BRAF mutations are V600E and this mutation is predominant in melanoma (5). This discovery raised the possibility that these tumors may be amenable to targeted therapy using inhibitors of BRAFV600E (5, 38). Recent clinical trial results with PLX4032, a BRAFV600E-specific inhibitor, are promising although it is still too early to calculate the overall survival of the treated patients (38). However, PLX4032 has been reported to activate MEK–ERK mitogen-activated protein kinase (MAPK) signaling in cells with BRAF wild-type kinase (39–41). This MAPK activity drives the uncontrolled proliferation of melanoma cells by upregulating expression of cyclin D1 and the suppression of the CDK inhibitor p27 (35, 42). Moreover, PLX4032 administration generates resistant cell types by activating EGF receptor (EGFR) and platelet-derived growth factor-β (PDGF-β refs. 43, 44). Interestingly, a combination of EGFR inhibitors and MEK inhibitors can overcome the resistance induced by PLX4032 (43, 45). Because tumors are heterogeneous populations of cells, administration of selective inhibitors such as PLX4032 may aggravate tumor burden. Various CDK inhibitors, such as flavopiridol, have also been investigated for melanoma therapy, however, these inhibitors failed in clinical trials (46).

Numerous ongoing clinical studies are evaluating the effects of individual inhibitors for BRAF kinase and CDK4, not on both pathways simultaneously (47). In this study, we have shown that gossypin induces cytotoxicity in melanoma cells through concurrent inhibition of BRAFV600E kinase and CDK4, not on both pathways simultaneously (47). In our study, we have shown that gossypin induces cytotoxicity in melanoma cells through concurrent inhibition of BRAFV600E kinase and CDK4. To the best of our knowledge, gossypin is the only compound that concurrently inhibits both BRAFV600E kinase and CDK4 in melanoma cells. Recently, a combination therapy of selective inhibitors has been anticipated to yield better outcomes in patients with BRAF/CDK4 mutations (47).

Gossypin exhibited greater cytotoxicity in melanoma cells possessing the BRAFV600E mutation, suggesting that gossypin is more selective toward BRAFV600E than BRAF wild-type kinase. This was confirmed by: (i) higher in vitro kinase inhibition of gossypin for BRAFV600E than BRAF wild-type kinase; (ii) hypophosphorylation of the MEK–ERK pathway by gossypin treatment in 1205Lu

or 100 mg/kg; Fig. 6A). Gossypin at 10 mg/kg was effective in reducing tumor burden by 36% when compared with PLX4032 (P < 0.05) and 45% when compared with roscovitine (P < 0.004). Although, the concentration of gossypin (100 mg/kg) was higher compared with PLX4032, a better reduction in tumor volume was noticed in this group. Gossypin at 100 mg/kg inhibited tumor growth even more effectively, by 62% and 68% when compared with PLX4032 (P < 0.03) and roscovitine (P < 0.004), respectively. It should be noted that any indications of toxicity such as weight loss or weakness was not found in this group.

Immunohistochemical analysis of tumor tissues from gossypin-treated animals exhibited a significant decline in the phosphorylation levels of MEK and ERK, decreased expression of Ki67, and increased number of apoptosis cells by TUNEL assay, when compared with PLX4032 or roscovitine-treated groups (Fig. 6B). This confirms our in vitro studies that gossypin could attenuate BRAFV600E-mediated phosphorylation of MEK–ERK signaling pathway and tumor cell proliferation in melanoma. Consistent with this, Western blot analysis of the tumor samples indicate decreased phosphorylation of MEK, ERK, Rb, and reduction in cyclin D1 protein levels in gossypin-treated groups compared with vehicle-treated groups (Fig. 6C). Because the number of apoptotic cells in gossypin-treated animals was increased, we tested whether a caspase-mediated mechanism is involved in apoptosis. To test this, caspase-3/7 activity in tumor tissues from control-, gossypin-, PLX4032-, and roscovitine-treated animals were measured. Caspase-3/7 activity in tumor tissues from gossypin-treated animals was significantly higher than control as well as PLX4032- or roscovitine-treated animals, indicating a caspase-3/7-mediated apoptosis in response to gossypin (Fig. 6D).

Figure 5. Gossypin docked in the active site of BRAFV600E (A) and CDK4 (B). Gossypin and surrounding residues are shown in thick lines and the hydrogen bonds are shown in broken lines (left). The binding mode of gossypin (represented by thick lines) in the active site of protein (represented by surface model) is shown at the right.
cells and A375 cells; (iii) failure of gossypin to inhibit the MEK–ERK pathway in SKML31 cells; and (iv) specific binding of gossypin to BRAFV600E kinase but not to BRAF wild-type kinase, as shown by molecular docking studies.

Gossypin did not affect MEK–ERK signaling in melanoma cells with BRAF wild-type kinase, suggesting that gossypin may not be protumorigenic, unlike PLX4032. Our study clearly identified CDK4 as a direct target of gossypin in melanoma cells with BRAF wild-type kinase by: (i) attenuated phosphorylation of Rb and decreased expression of cyclin D1 by gossypin in SKMEL-31 cells; (ii) in vitro kinase inhibition of CDK4 by gossypin; and (iii) binding of gossypin in the ATP-binding pocket of CDK4 as determined by docking studies. Previous docking studies identified CDK2 as a direct target of gossypin (48). Gossypin exhibited approximately 75% to 80% inhibition for CDK4 at 150 μmol/L, suggesting that CDK4 is the specific target of gossypin (Fig. 3E). This result was further supported by the finding that gossypin impedes...
G1/S transition in the cell cycle (Fig. 3A–D), which is a reflection of CDK4 but not CDK2 inhibition. Under normal conditions, CDK4 forms a complex with cyclin D1 leading to the activation of E2F target genes, including cyclin D1, through phosphorylation of Rb (49). Therefore, the observed cytotoxicity of gossypin in SKMEL-31 could be a consequence of the combined effect of CDK4 kinase inhibition and decreased expression of cyclin D1 (Fig. 5B).

In agreement with our in vitro studies, gossypin inhibited tumor growth through inhibition of MEK–ERK pathway. Similarly, apoptotic effect of gossypin in vitro was also observed in our in vitro studies. In summary, gossypin reduced tumor growth by inhibiting MEK–ERK pathway and through induction of apoptosis by activating caspase-3/7 effector molecules. Previously, it has been reported that gossypin induced apoptosis in myeloma cells by activating caspase-3/7 apoptotic pathway and our current finding is consistent with this observation (22).

Even though crystal structure of gossypin bound to BRAFV600E kinase or CDK4 is not presented, docking of gossypin suggests flavonol skeleton binds to the active site of these proteins and the results of our cell culture study, together with biochemical, docking, and follow-up xenograft studies, provide strong evidence for the therapeutic value of gossypin in treating melanoma. Selective BRAF inhibitors may cause adverse effects in some patients, as melanomas in different people may harbor different mutations. At the moment, there is no single therapeutic inhibitor may cause adverse effects in some patients, as melanomas in different people may harbor different mutations. At the moment, there is no single therapeutic inhibitor may cause adverse effects in some patients, as melanomas in different people may harbor different mutations. At the moment, there is no single therapeutic inhibitor may cause adverse effects in some patients, as melanomas in different people may harbor different mutations. At the moment, there is no single therapeutic

our results open a new avenue for the generation of a new class of compounds for the treatment of melanoma.

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

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References


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Gossypin as a Novel Selective Dual Inhibitor of v-raf Murine Sarcoma Viral Oncogene Homolog B1 and Cyclin-Dependent Kinase 4 for Melanoma

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