Specific targeting of Wnt/β-catenin signaling in human melanoma cells by a dietary triterpene lupeol

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Wingless (Wnt) signaling pathway regulates a variety of cellular processes including proliferation, differentiation, survival, apoptosis and cell motility. Abrupt activation of Wnt/β-catenin pathway has been observed in approximately one-third of melanomas and this subset has very poor prognosis suggesting that targeting Wnt signaling could be a promising strategy against this subtype. Mel 928 and Mel 1241 melanoma cells representative of cells with constitutive activation of Wnt/β-catenin signaling pathway and Mel 1011 representative of cells that lack this pathway were treated with a dietary triterpene lupeol and its effects on growth, proliferation, β-catenin transcriptional activity and Wnt target genes were determined both in vitro and in vivo. Lupeol treatment to Mel 928 and Mel 1241 but not Mel 1011 cells resulted in a dose-dependent (i) decrease in cell viability, (ii) induction of apoptosis, (iii) decrease in colchonogenetic potential, (iv) decrease in β-catenin transcriptional activity and (v) decrease in the expression of Wnt target genes. Most importantly, lupeol restricted the translocation of β-catenin from the cytoplasm to the nucleus. Lupeol also decreased the growth of Mel 928 but not Mel 1011-derived tumors implanted in the athymic nude mice. The decrease in Mel 928-derived tumor growth was associated with a decrease in the expression of Wnt target genes c-myc, cyclin D1, proliferation markers proliferating cell nuclear antigen and Ki-67 and invasion marker osteopontin. We suggest that lupeol alone or as an adjuvant to current therapies could be developed as an agent for the management of human melanomas harboring constitutive Wnt/β-catenin signaling.

Introduction

Melanoma, the most serious form of skin cancer, is curable in its initial stages but when detected at later stages, is one of the most lethal malignancies (1,2). The increasing incidence of melanoma and its poor prognosis advocates the need for the development of novel preventive approaches such as the use of specific targeting agents derived from natural products (3). Lupeol [Lup-20(29)-en-3β-ol], a dietary triterpene, found in various fruits, vegetables and some medicinal herbs (4–8) has been shown to possess strong anti-inflammatory, anticarcinogenic, anti-mutagenic and anti-malarial activity both in vitro and in vivo (5,7,9–15). Our recent studies have also shown that lupeol possesses chemopreventive effects in a two-stage mouse skin carcinogenesis model (16).

Wnt/β-catenin signaling pathway is reported to play an important role in embryogenesis, stem cell maintenance and tumorigenesis, including melanoma progression (17–19). β-catenin is a key component of the Wnt signaling pathway and its translocation to the nucleus initiates transcription of downstream target genes (20). Overexpression of Wnt ligands, mutations in components of Wnt pathway and stabilization of β-catenin are the most common alterations associated with constitutive activation of Wnt signaling pathway and are observed in a variety of cancers including melanomas. Melanoma cells exhibit differential β-catenin expression with Mel 1241 cell line carrying a stabilizing mutation of β-catenin, whereas Mel 928 expresses wild-type β-catenin without detectable adenomatosis polyposis coli (APC). Mel 1011 cells harbor both intact β-catenin and APC. Mel 928 and Mel 1241 cells are also characterized by nuclear localization of β-catenin and constitutive activation of β-catenin/Tcf-dependent transcription (21,22). It is noteworthy that the hallmark of active Wnt signaling, nuclear localization of β-catenin, has been observed in about a third of primary and metastatic human melanomas (21,23).

Recently, we demonstrated that lupeol inhibits the growth of metastatic melanoma cells in vitro and in vivo (24). While defining the mechanism of these inhibitory effects of lupeol on melanoma cells, we made a novel observation that it specifically targets melanoma cells that harbor constitutive Wnt/β-catenin signaling pathway. This information coupled with our previously published data (24), which demonstrated that lupeol exerts negligible toxicity on normal human melanocytes, advocates that lupeol could be developed as a potential agent for the management of melanomas that harbor constitutive Wnt/β-catenin signaling pathway.

Materials and methods

Materials

The plasmids dominant-negative T-cell factor (TCF) 4, β-cateninS33Y, pcDNA and TOP-Flash reporter plasmid bearing the TCF4-binding sequence were obtained from Drs K.Kinzler and B.Vogelstein (25–28), whereas pTK-puro plasmid was obtained from Addgene (Cambridge, MA). Renilla luciferase (pRL-TK) plasmid was obtained from Promega (Madison, WI). Precast 12% Tris-glycine polyacrylamide gels were obtained from Invitrogen (Carlsbad, CA). Antibodies against proliferating cell nuclear antigen (PCNA), Ki-67, osteopontin, β-catenin, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) 2/9 antibodies that detect activated proteins were obtained from Cell Signaling Technology (Danvers, MA) and BD Biosciences Pharmingen (San Jose, CA), respectively. Pathscan® ELISA kit for β-catenin was obtained from Cell Signaling Technology.

Cell lines and cell culture

The melanoma cell lines Mel 928, Mel 1241 and Mel 1011 were kindly provided by Dr Paul Robbins (Center of Cancer Research, National Cancer Institute, Bethesda, MD) (22). Cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution, containing penicillin, streptomycin and amphotericin B (PSM) under standard growth conditions (5% CO2, 37°C, humidified atmosphere). The 293T cells were obtained from the American Type Culture Collection, Manassas, VA.

Treatment of cells with lupeol

A stock solution of lupeol (10 mM) was prepared by dissolving it in warm ethanol and diluting in dimethyl sulfoxide in a 1:1 ratio. For dose-dependent studies, the cells (50% confluent) were treated with lupeol (20–60 μM) for 48 h in complete cell media. All treatment protocols and controls were conducted as described previously (24).

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cells were screened against puromycin (1.25 μg/ml). Forty-eight hours after transfection, the cells were treated with lupeol as described earlier and the lupeol treatment was repeated every 2 days. Seven to ten days posttransfection, the colonies formed were fixed, stained with crystal violet, and counted. Each bar represents percent colonies, where colonies in untreated cells were regarded as 100%. *P < 0.01 compared with vehicle-treated controls; #P < 0.05 with 20 μM treatment; ##P < 0.01 compared with 20 μM treatment (one-way analysis of variance followed by Tukey’s HSD test).

**Annexin V staining**

The annexin V-FLUOS staining kit (Roche Applied Biosciences, Indianapolis, IN) was used for the detection of apoptotic cells. Assay was performed as described (29).

**Luciferase reporter assay**

Using Lipofectamine-2000 reagent, melanoma cells were transfected as per vendor’s protocol (Invitrogen). Fresh media containing lupeol (40 and 60 μM) was added 12 h post-transfection. After 24 h, the cells were harvested and transcripational activity was measured in terms of luciferase activity by using the dual-luciferase reporter assay system (Promega) as per manufacturer’s recommendations. The transcriptional activity was measured in a dose- and time-dependent manner.

**Preparation of cell lysates for immunoblot analysis**

Whole cell, cytosolic and nuclear lysates were prepared and western blotting was performed as described earlier (24,29).

**Enzyme-linked immunosorbent assay**

β-catenin ELISA kit was used for the quantitative analysis of β-catenin in cytosolic and nuclear lysates as per vendor’s protocol. The absorbance was
Fig. 2. Effect of lupeol on Wnt/β-catenin signaling in melanoma cells. (A) TOP-Flash luciferase assay: melanoma cells were treated as indicated in Materials and Methods. The β-catenin/Tcf responsive luciferase activity was measured and normalized against renilla luciferase activity. Each bar represents relative luciferase activity upon lupeol treatment ± SE. *P < 0.01 compared with vehicle-treated controls (one-way analysis of variance followed by Tukey’s honestly significant difference test). (B) Protein expression of Wnt target genes in melanoma cells: protein expression of coding region determinant-binding protein (CRD-BP), microphthalmia-associated transcription factor (MITF) and Cyclin D1; numbers on top represent relative density normalized to β-actin. (C and D) Localization of β-catenin: cytosolic and nuclear protein expression of β-catenin. Numbers on top represent relative density normalized to β-actin. All the immunoblots shown here are representative of three independent experiments with similar results.

Immunocytostaining
Melanoma cells were cultured on chambered slides and treated with lupeol as indicated previously. The cells were fixed in cold acetone for 5 min and blocked with 2.5% normal serum. Slides were incubated overnight with anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by fluorescence-tagged secondary antibody, covered with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen) and visualized using Nikon Eclipse Ti microscope. Images were captured with an attached camera.

Tumorigenicity studies in athymic nude mice
Athymic (nu/nu) nude mice (male; 4–6 weeks old) were obtained from National Cancer Institute and housed in the animal care facility at The University of Wisconsin School of Medicine and Public Health under pathogen-free conditions with 12 h light–dark schedule) and fed with autoclaved Harlan Teklad sterilizable rodent diet (Harlan, Madison, WI) and water ad libitum. We determined the efficiency of lupeol in two settings reflective of chemopreventive and chemotherapeutic protocols. Twenty-four athymic nude mice were implanted with human melanoma cells that harbor (Mel 928) or lack (Mel 1011) constitutive Wnt/β-catenin signaling. Melanoma cells (10^6) were suspended in a 50 μl media and 50 μl Matrigel (BD Biosciences Pharmingen) and inoculated subcutaneously on the left and right flanks of athymic nude mice. On the following day, the animals were randomly divided into three groups (control, chemopreventive and chemotherapeutic protocols).

For the chemopreventive approach, the animals received an intraperitoneal injection of lupeol (40 mg/kg body wt) in 200 μl of corn oil 24 h postinoculation. This dose was used successfully in our prior studies and is physiologically attainable and is non-toxic (24). For the therapeutic approach, the treatment with lupeol was initiated when the tumor volume reached ~150 mm^3. This group of animals received intraperitoneal injection of lupeol (40 mg/kg body wt) in 200 μl of corn oil. The control group received corn oil alone, which was initiated 24 h postinoculation. All treatments with lupeol were performed thrice a week until the termination of the experiment. The animals were monitored closely for tumor growth. The treatment schedule was continued until the tumors in the control group reached a volume of ~800 mm^3. At this time, the animals were withdrawn from the study and euthanized. The tumors were excised, snap frozen in liquid nitrogen and kept at −80°C for further biochemical analysis. Throughout the experiment, the animals were monitored for possible treatment-associated toxicity and changes in body weight. Tumor size was measured by determining two perpendicular dimensions and the height using vernier caliper. The tumor volume was calculated using the formula V = 1/2(4π/3) (L1/2) (L2/2) (H) = 0.5238 L1L2H, where L1 is the long diameter, L2 is short diameter and H is the height (30).

Preparation of tumor lysates
For immunoblotting analysis, a portion of the tumor tissues was thawed on ice and homogenized using a hand held homogenizer in 700 μl ice-cold lysis buffer as described earlier (24). The tumor lysates were stored at −80°C. The protein concentration was determined by the BCA protein assay kit using the manufacturer’s protocol (Pierce, Rockford, IL).

Immunohistochemical analysis
Immunohistochemical staining was performed as described earlier (24,31). Paraffin-embedded sections were de-waxed, rehydrated, blocked and incubated overnight with primary antibody followed by subsequent secondary for 1 h. All the antibodies were procured from Santa Cruz Biotechnology. The stained slides were dehydrated, mounted in Permount and visualized using Nikon Eclipse Ti microscope. Images were captured with a camera attached to the microscope.

Densitometry analysis
Immunoblots were scanned by HP Precisionscan Pro 3.13 (Hewlett-Packard Co., Palo Alto, CA). Densitometry measurements of the scanned bands were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT). Data were normalized to β-actin or suitable loading controls and expressed as mean ± SEM followed by appropriate statistical analysis.

Statistical analysis
Data were analyzed for statistical significance with the use of analysis of variance followed by Tukey’s honestly significant difference test. Values of
Results

Effect of lupeol on human melanoma cell viability, induction of apoptosis and colonogenic potential

Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, we first evaluated the effect of lupeol (20–60 µM; 48 h) on cell viability. Lupeol treatment resulted in a significant dose-dependent decrease in the viability of Mel 928 and Mel 1241 cells that exhibit constitutively active Wnt/β-catenin signaling. These effects were absent in Mel 1011 cells that lack constitutively active Wnt/β-catenin signaling (Figure 1A). The IC_{50} values for Mel 928, Mel 1241 and Mel 1011 were 75, 72 and 135 µM, respectively.

We next determined the effect of lupeol treatment on caspase 3/7 activity in melanoma cells. Lupeol treatment caused a dose-dependent increase in caspase 3/7 activity in Mel 928 and Mel 1241 cells, whereas no significant change was observed in Mel 1011 cells (Figure 1B). We next determined the effect of lupeol on induction of apoptosis in melanoma cells. We observed increased staining of annexin V in Mel 928 cells, whereas minimal staining was observed in Mel 1011 cells indicating that lupeol treatment resulted in increased apoptosis in Mel 928 cells but not in Mel 1011 cells (Figure 1C).

Next, we asked whether treatment with lupeol could exert greater activity on the formation of colonies, which allows an investigation over a longer period of time. More than 50% decrease in the number of colonies was observed in Mel 928 and Mel 1241 cells, whereas no significant decrease in the number or size of colonies was observed in Mel 1011 cells treated with lupeol (Figure 1D).

Effect of lupeol on Wnt/β-catenin signaling in melanoma cells

In our next set of experiments, we tested whether lupeol could inhibit the transcriptional activity of Wnt/β-catenin signaling which in turn would lead to inhibition of downstream targets. In Mel 928 and Mel 1241 cells treated with lupeol (40 and 60 µM) for 24 h, we observed 50% decrease in β-catenin transcriptional activity (Figure 2A), an effect persistent for up to 48 h. Further, melanoma cells (Mel 1011, Mel 928 and Mel 1241) were treated with lupeol (40–60 µM) for 48 h and the protein expression of various Wnt target genes was evaluated. In Mel 928 and Mel 1241 cells, we observed a dose-dependent decrease in Wnt target genes such as coding region determinant-binding protein, microphthalmia-associated transcription factor and cyclin D1, whereas no decrease in Wnt target genes was observed in Mel 1011 cells treated with lupeol (Figure 2B). We also investigated the localization of β-catenin after lupeol treatment. In melanoma cells (Mel 928 and Mel 1241), we observed a decrease in β-catenin expression in the nucleus with a corresponding increase in cytosolic β-catenin, whereas no change in the localization of β-catenin was observed in Mel 1011 cells (Figure 2C and D). We further performed enzyme-linked immunosorbent assay and immunofluorescence analysis and observed a decrease in cytosolic-to-nuclear localization of β-catenin in melanoma cells that exhibit constitutively active Wnt signaling (Mel 928 and Mel 1241 cells), whereas no significant change in β-catenin localization was observed in Mel 1011 cells that

P < 0.05 were considered to be statistically significant. For in vivo experiments, survival estimates were performed by ‘R-Project’ for statistical computing.

Fig. 3. Effect of lupeol on β-catenin expression in melanoma cells. (A and B) Nuclear and cytosolic fractions of lupeol-treated melanoma cells were analyzed by enzyme-linked immunosorbent assay specific for β-catenin. The readings shown here are representative of three independent experiments with similar results. Bars ± SE. *P < 0.05 compared with vehicle-treated controls (one-way analysis of variance followed by Tukey’s honestly significant difference test). (C) Photomicrographs showing β-catenin localization in melanoma cells treated with lupeol. The data shown here is representative of three independent experiments with similar results.
do not harbor constitutive activation of Wnt/β-catenin signaling (Figure 3).

**Effect of lupeol administration on tumor growth in athymic nude mice**

To establish the in vivo relevance of our in vitro data, we determined and compared the efficacy of lupeol on the tumor growth of melanoma cells differing in Wnt/β-catenin signaling activity implanted in nude mice. As shown in Figure 4A, significant inhibition in tumor growth and ultimate tumor burden was observed with lupeol treatment in nude mice that were implanted with Mel 928 cells. At the termination of the experiment, lupeol treatment (therapy group) resulted in a 35% reduction in tumor volume at a dose of 40 mg/kg body wt as compared with untreated controls. In the lupeol-preventive group, the treatment resulted in a 50% decrease in tumor burden as compared with vehicle-treated controls. The differences between the two treatment groups were not statistically significant. At the termination of the experiment at 9 weeks on test, the average tumor volume in control, lupeol treatment and lupeol-preventive groups was ~800, 520 and 460 mm³, respectively (Figure 4A). No significant decrease in tumor volume was observed in mice implanted with Mel 1011 cells (Figure 4B). We also observed that the rate of growth of Mel 928-derived tumors was higher than Mel 1011-implanted tumors. It is important to mention that the volume at which the experiment was terminated in Mel 1011-implanted tumors is low because a majority of the Mel 1011-implanted tumors ruptured during the course of the experiment. To assess the effects of treatments on toxicity, body weight and food-water consumption were monitored throughout the study. Lupeol was not found to be associated with any apparent toxicity in terms of food and fluid consumption as well as body weight (data not shown).

We further performed biochemical analysis in tumors excised at the termination of the experiment. As shown in Figure 5A and B, lupeol treatment resulted in a decrease in expression levels of Wnt target genes (c-myc and cyclin D1) in Mel 928-implanted tumors, whereas no change in the expression of Wnt target genes was observed in Mel 1011-implanted tumors. Immunohistochemical analysis of tumors also indicated a decreased nuclear localization of β-catenin in Mel 928-implanted tumors that were derived from lupeol-treated animals (Figure 5C). No change in β-catenin localization was observed in Mel 1011-implanted tumors. Since lupeol treatment was observed to decrease the tumorigenic potential of Mel 928 cells in vivo, we next investigated the effect of lupeol administration on the expression level of PCNA, osteopontin and Ki-67 proteins, which are known markers of proliferation. Decreased expression of PCNA, osteopontin and Ki-67 was observed in tumors harvested from lupeol-treated mice and no significant decrease in expression of proliferative markers was observed in Mel 1011-implanted tumors (Figure 5D). A recent study suggests that blockade of Wnt/β-catenin signaling inhibits angiogenesis and tumor growth (32). Since angiogenesis plays a significant role in cancer progression, our next aim was to determine the effect of lupeol treatment on key modulators of angiogenesis. By immunoblot analysis, we observed a 13-fold decrease in the expression of VEGF in tumors of lupeol-treated animals (Figure 5E). We also observed that lupeol-treated animals had a decrease in MMP2 and MMP9 and a corresponding increase in their inhibitors [tissue inhibitors of matrix metalloproteinase (TIMP) 1 and TIMP2], decreasing the MMP:TIMP ratio by at least 10-fold (Figure 5E). No significant modulation of these proteins was observed in Mel 1011-implanted tumors upon lupeol treatment.

**Effect of lupeol on the colonogenic potential of melanoma cells upon modulation of Wnt/β-catenin signaling pathway**

In our next set of experiments, we tested the efficacy of lupeol upon modulation of Wnt/β-catenin signaling. We investigated the effect of lupeol on the colonogenic potential of melanoma cells with silenced Wnt signaling. Our data demonstrated that when Wnt signaling was inhibited (by dominant-negative TCF4), the efficacy of lupeol was lost and no significant decrease in number of colonies was observed (Figure 6A). For our next set of experiments, we artificially induced Wnt signaling in Mel 1011 cells (that lack Wnt signaling). This was achieved by transfecting Mel 1011 cells with β-catenin carrying stabilizing mutation (β-cateninS33Y). Lupeol treatment to these cells resulted in a dose-dependent inhibition in the number of colonies formed, suggesting that activation of Wnt/β-catenin signaling sensitizes these melanoma cells to lupeol (Figure 6B).

**Effect of lupeol on Wnt/β-catenin signaling in 293T cells transfected with stable β-catenin**

In our final set of experiments, we induced Wnt/β-catenin signaling in 293T cells and tested the effect of lupeol on β-catenin transcriptional activity and expression of Wnt target genes. The rationale for using 293T cells is that they lack constitutively active Wnt/β-catenin signaling. Activating the pathway using stable β-catenin, upon lupeol treatment, we observed a dose-dependent decrease in β-catenin transcriptional activity (TOP-Flash luciferase assay) (Figure 6C). In these cells, a dose-dependent decrease in expression of Wnt target genes, viz c-myc and cyclin D1 was also observed (Figure 6C).

**Discussion**

The most significant and novel finding in this study is the demonstration that a non-toxic dietary triterpene lupeol specifically targets melanoma cells that harbor constitutive activation of Wnt/β-catenin
Fig. 5. Effect of lupeol administration on the expression level of Wnt targets, β-catenin localization, proliferation markers and molecules involved in angiogenesis under \textit{in vivo} conditions. (A) Protein expression of c-myc and cyclin D1 in Mel 928- and Mel 1011-implanted tumors. The immunoblots shown here are representative of two or four tissue samples randomly selected from each group. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. (B) Histogram showing relative density normalized to β-actin of c-myc and cyclin D1 in Mel 928 cell-implanted tumors. Data is mean ± SE of the data represented above. *$P < 0.05$ compared with vehicle-treated controls. (C) Photomicrographs showing expression of β-catenin by immunohistochemical analysis. Immunostaining data was confirmed in two slides each from four animals. (D) Protein levels of PCNA, osteopontin and Ki 67 as determined by immunohistochemical analysis. Immunostaining data was confirmed in two slides each from eight animals. (E) Protein expression level of VEGF, MMP2, MMP9, TIMP1 and TIMP2 in Mel 928- and Mel 1011-implanted tumors. The immunoblots shown here are representative of two or four tissue samples randomly selected from each group. Histogram showing ratios of MMP:TIMP in Mel 928 and Mel 1011 cell-implanted tumors. Data is mean ± SE of the data represented above. *$P < 0.05$ compared with vehicle-treated controls.
signaling. The prognosis of melanoma patients with distinct metastasis remains poor and in general is not significantly influenced by any treatment intervention because metastatic melanomas are inevitably resistant to conventional therapeutic agents. Several natural agents, including lupeol, with high anticancer efficacy and relatively minimal toxicity to normal tissues are suggested as possible candidates for chemoprevention of melanoma (3, 24, 33–35).

In vitro as well as in vivo studies from our laboratory and elsewhere have suggested that lupeol could impart chemopreventive as well as chemotherapeutic effects against various cancer types (13, 16, 24, 36). In an earlier study (24), we demonstrated the anticancer efficacy of lupeol against metastatic melanoma 451 Lu cells. Since metastatic potential of melanomas is associated with elevated expression of Wnt/β-catenin signaling pathway, we wanted to test the role of lupeol in cells that express differential expression of Wnt/β-catenin pathway. In this current study, we provide compelling evidence that lupeol is significantly more effective against melanomas that exhibit constitutive activation of Wnt/β-catenin signaling as compared with melanomas that lack constitutively active Wnt/β-catenin signaling. We employed Mel 1241 cell line that carries a stabilizing mutation of β-catenin and Mel 928 that expresses wild-type β-catenin, but no detectable APC. Mel 1011, containing both intact β-catenin and APC are used as control. Mel 928 and Mel 1241 cells are characterized by nuclear localization of β-catenin and constitutive activation of β-catenin/TCF-dependent transcription (21, 22).

Our data suggests that lupeol treatment causes a dose-dependent decrease in viability and an increase in apoptosis (activation of caspases and expression of annexin V) in melanoma cells that exhibit constitutive activation of Wnt/β-catenin signaling as compared with melanomas that lack constitutively active Wnt/β-catenin signaling. We employed Mel 1241 cell line that carries a stabilizing mutation of β-catenin and Mel 928 that expresses wild-type β-catenin, but no detectable APC. Mel 1011, containing both intact β-catenin and APC are used as control. Mel 928 and Mel 1241 cells are characterized by nuclear localization of β-catenin and constitutive activation of β-catenin/TCF-dependent transcription (21,22).

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The importance of cyclin D1 and c-myc (known Wnt target genes) (27, 45) as therapeutic targets for melanoma emanates from studies demonstrating that inhibiting the expression of cyclin D1 and c-myc causes apoptosis and shrinkage of tumors in mice (27, 45–48). It is noteworthy that lupeol treatment significantly decreases the expression level of c-myc and cyclin D1 in Mel 928-originated tumors. This data provides significant evidence that lupeol modulates Wnt signaling and lowers the expression of Wnt targets both in vitro and in vivo.
Fig. 6. Modulation of Wnt/β-catenin signaling pathway alters the efficacy of lupeol on the colonogenic potential of melanoma cells. (A) Wnt signaling was downregulated in melanoma cells (Mel 928 and Mel 1241) using dominant-negative TCF4. The cells were screened against puromycin and treated with lupeol. The colonies formed were stained as described in Materials and Methods. The crystal violet was dissolved in 50% acetic acid and absorbance was measured at 540 nm. The data is mean ± SE from a representative experiment repeated thrice with similar results. \( P < 0.05 \) compared with vehicle-treated controls.

(B) Mel 1011 cells were transfected with stable β-catenin (β-cateninS33Y) to induce Wnt/β-catenin signaling. The transfected cells were selected for puromycin resistance and treated with lupeol. The colonies formed were stained with crystal violet. The crystal violet was dissolved in 50% acetic acid and the absorbance was read at 540 nm. The data is mean ± SE from a representative experiment repeated thrice with similar results. \( P < 0.05 \) compared with vehicle-treated controls.

(C) TOP-Flash reporter assay in 293 T cells transfected with stable β-catenin and treated with lupeol. β-cateninS33Y-transfected 293T cells were treated with lupeol. The β-catenin/Tcf-responsive luciferase activity was measured and normalized against renilla luciferase activity. Data is mean ± SE of 3 samples. \( P < 0.05 \) compared with vehicle-treated controls. Effect of lupeol treatment on the expression level of important Wnt target genes c-myc and cyclin D1 in Wnt-activated-293T cells. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin.
A discernable decrease in expression of proliferation markers such as PCNA, osteopontin and Ki-67 was also observed in lupeol-treated Mel 928-implanted tumors suggesting decreased proliferative activity upon lupeol treatment. Recently, there has been an increased interest in understanding the angiogenesis machinery that causes chemoresistance in melanoma cells (49,50). VEGF is an important signaling protein and plays an essential role in angiogenesis (49). The role of MMPs in invasion and metastasis in melanomas and other malignancies has been extensively studied (51,52). MMP activity is modulated by a family of naturally occurring TIMPs. In Mel 928-implanted tumors, we observed a 13-fold decrease in expression levels of VEGF in lupeol-treated animals versus those treated with corn oil only. A 10-fold decrease in ratios of MMP9/TIMP1 and MMP2/TIMP2 was observed in Mel 928-implanted lupeol-treated tumors. Thus, our data suggest that the effect of lupeol on key regulators of angiogenesis (MMPs and TIMPs) is very significant.

Furthermore, we wanted to test the efficacy of lupeol when Wnt/β-catenin signaling is silenced. Colony formation assay reveals that upon silencing Wnt/β-catenin signaling, the efficacy of lupeol was lost suggesting that Wnt signaling is the key player for lupeol-induced effects. On the other hand, induction of Wnt signaling in Mel 1011 cells results in reduced colonogenic potential upon lupeol treatment, further verifying the Wnt-specific role of lupeol. In our conclusive experiments, we induced Wnt/β-catenin signaling in non-tumorigenic 293T cells. We have demonstrated that lupeol treatment resulted in a dose-dependent decrease in β-catenin transcriptional activity and protein expression of Wnt target genes (27,45).

In summary, we have demonstrated the effect of lupeol against Wnt/β-catenin signaling network resulting in decrease in proliferation, viability and growth of melanoma cells and tumors in mice. Taken together, our present findings demonstrate the anticancer efficacy of lupeol against melanomas that exhibit constitutively active Wnt/β-catenin signaling. These observations coupled with our earlier published work showing lack of effect of lupeol on normal human melanocytes (24) suggest further in depth studies to understand the mechanism behind the modulation of Wnt/β-catenin signaling by lupeol. The positive outcomes of such studies could form a strong basis for the development of lupeol as a novel agent for malignancies that exhibit constitutive Wnt signaling that occurs in a third of melanomas that have poor prognosis.

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References

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Specific targeting of Wnt/b-catenin by lupeol