*Cancer Therapy: Preclinical*

# Lupeol Inhibits Growth of Highly Aggressive Human Metastatic Melanoma Cells *In vitro* and *In vivo* by Inducing Apoptosis

Mohammad Saleem, Nityanand Maddodi, Mohammad Abu Zaid, Naghma Khan, Bilal bin Hafeez, Mohammad Asim, Yewseok Suh, Jung-Mi Yun, Vijayasaradhi Setaluri, and Hasan Mukhtar

**Abstract**

**Purpose:** Poor prognosis of metastatic melanoma mandates the development of novel strategies for its treatment and prevention. In this study, the effect of lupeol, a diet-based triterpene, was determined on the growth and tumorigenicity of human melanoma cells *in vitro* and *in vivo*.

**Experimental Design:** Normal human melanocytes, and human metastatic (451Lu) and nonmetastatic (WM35) cells were treated with lupeol; its effect on growth, proliferation, and apoptosis were evaluated. Further athymic nude mice bearing 451Lu cell–originated tumors were administered with lupeol thrice a week, and its effect on tumor growth and surrogate biomarkers was evaluated.

**Results:** Lupeol significantly decreased the viability of 451Lu and WM35 melanoma cells but had only a marginal effect on normal human melanocyte cells at similar doses. Lupeol treatment of 451Lu cells caused (*a*) $G_1$-S phase cell cycle arrest and apoptosis; (*b*) down-regulation of Bcl2 and up-regulation of Bax; (*c*) activation of caspase-3 and induction of poly(ADP)ribose polymerase cleavage; (*d*) decreased expression of cyclin D1, cyclin D2, and cdk2; and (*e*) increased expression of p21 protein. Next, lupeol significantly reduced 451Lu tumor growth in athymic nude mice and modulated the expression of proliferation markers, apoptotic markers, and cell cycle regulatory molecules in tumor xenografts.

**Conclusion:** Our findings showed the anticancer efficacy of lupeol with mechanistic rationale against metastatic human melanoma cells. We suggest that lupeol, alone or as an adjuvant to current therapies, could be useful for the management of human melanoma.

Melanoma, a malignant neoplasm of melanocytes, is recognized as one of the most aggressive cancers with relatively high propensity for metastasis. In the United States alone, 59,940 newly diagnosed invasive melanomas will be detected and 8,110 deaths are anticipated in 2007, making melanoma the fifth most common invasive cancer in men and sixth most common in women (1). The incidence of melanoma continues to increase despite public health initiatives that have promoted sun protection. In the United States, its incident has increased 15-fold in the past 40 years, a rate more rapid than known for most other malignancies. Unfortunately, strategies devoted solely to protecting against UV radiation have, at best, had only a modest effect on the development of melanoma and standard systemic therapies for melanoma such as IFN remains unsatisfactory. The increasing incidence of melanoma and its poor prognosis in advanced stages mandate the development of novel approaches for its prevention through chemoprevention. Chemoprevention by naturally occurring agents present in food and beverages consumed by humans has shown benefits in certain cancers, including nonmelanoma skin cancers (2).

Unexpected observations from cardiovascular disease trials have fueled speculation that melanoma chemoprevention may be possible (3). Theoretically, there could be three different strategies for chemoprevention of melanoma (3). Primary chemoprevention would prevent occurrence of melanoma in healthy individuals. Secondary chemoprevention will aim to prevent premalignant melanoma precursors from becoming melanoma. Finally, tertiary chemoprevention will seek to prevent melanoma in patients with treated primary melanoma and no current signs of the disease (3). Recent epidemiologic studies have found a correlation between populations with higher consumption of selenium and vitamin E, fruits, and tomatoes, in lowering the risk of overall cancer in humans (4, 5). Consistent with this notion, several single natural agents, such as green tea polyphenol epigallocatechin gallate and resveratrol, are being studied as chemopreventive agents against human melanoma (6, 7).

Lupeol [Lup-20(29)-en-3β-ol] (Fig. 1), a triterpene found in fruits such as olive, mango, strawberry, grapes, and figs; in many vegetables; and in several medicinal plants (8). Lupeol is found as an active constituent of various medicinal plants used by native people in the treatment of various skin ailments in North America, Japan, China, Latin America, and Caribbean

islands (ref. 8 and references therein). Lupeol possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activity in *in vitro* and *in vivo* systems; acts as a potent inhibitor of protein kinases and serine proteases; and inhibits the activity of DNA topoisomerase II, a known target for anticancer chemotherapy (9–11). It has also been shown that lupeol induces differentiation of mouse melanoma cells (12). Recently, we have shown that lupeol exhibits significant antitumor-promoting activity in a two-stage model of mouse skin carcinogenesis (13).

Here, we show that lupeol induces cell cycle arrest followed by apoptosis of human metastatic melanoma cell line 451Lu cells and also inhibits its growth when propagated in a mouse xenograft model. We suggest that lupeol could be an effective agent against human melanoma.

## Materials and Methods

*Cell culture.* Normal human melanocytes (NHM), human primary melanoma cells WM35, and metastatic melanoma cells 451Lu were obtained from American Type Culture Collection. NHMs were grown in cascade medium 254 supplemented with phorbol 12-myristate 13-acetate–free human melanocytes growth supplement (Cascade Biologics). Melanoma cells were grown in 2% tumor medium containing a 4:1 mixture of MCDB 153 medium with 1.5 g/L sodium bicarbonate and Leibovitz's L-15 medium supplemented with 2 mmol/L L-glutamine, 0.005 mg/mL bovine insulin, 1.68 mmol/L $CaCl_2$, and 2% fetal bovine serum supplemented with 1% penicillin-streptomycin (Cellgro Mediatech, Inc.) at 5% $CO_2$ at 37°C under humidified atmosphere in an incubator.

*Treatment of cells.* A stock solution of lupeol (30 mmol/L) was prepared by dissolving it in warm alcohol and diluting in DMSO in a 1:1 ratio. For dose-dependent studies, the cells (50% confluent) were treated with lupeol (40-80 μmol/L) for 72 h in complete cell medium. The final concentrations of DMSO and alcohol were 0.25% and 0.075%, respectively, in all treatment protocols. In preliminary experiments, we found that these concentrations of DMSO or alcohol had no effect on cell growth. Vehicle-treated cells served as controls. After 72 h of treatment with lupeol, the cells were harvested and cell lysates were prepared and stored at -80°C for later use.

*Cell viability assay.* The effect of lupeol on the viability of NHM, 451Lu, and WM35 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT87) assay. The cells were plated at $1 \times 10^4$ per well in 200 μL of complete culture medium and treated with 20 to 80 μmol/L concentrations of lupeol in 96-well microtiter plates. After incubation for 72 h at 37°C in a humidified incubator, cell viability was determined. MTT (5 mg/mL in PBS) was added to each well and incubated for 2 h after which the plate was centrifuged at 1,800 rpm for 5 min at 4°C. After careful removal of the medium, 0.1 mL of buffered DMSO was added to each well and the plates were shaken. The absorbance was recorded at the wavelength of 540 nm. The effect of lupeol on growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as 100% viable.

*Western blot analysis.* Cell and tissue lysates were prepared in cold lysis buffer [0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mol/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L $Na_3VO_4$, 0.5% NP40, 1% Triton X-100, 1 mol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The lysate was collected and cleared by centrifugation, and the supernatant was aliquoted and stored at -80°C. The protein content in the lysates was measured by BCA protein assay (Pierce), as per the manufacturer's protocol. For Western blot analysis, 40 μg of protein were resolved over 12% Tris-glycine polyacrylamide gels (Novex) under nonreduced conditions, transferred onto nitrocel-

lulose membranes, and subsequently incubated in blocking buffer [5% nonfat dry milk/1% Tween 20; in 20 mmol/L TBS (pH 7.6)] for 2 h. The blots were incubated with appropriate primary antibody, washed, and incubated with appropriate secondary horseradish peroxidase–conjugated antibody (Amersham Biosciences). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences) and autoradiography, using XAR-5 film (Eastman Kodak). Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin (Sigma).

*Quantification of apoptosis.* Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) method. After incubating cells with lupeol (40-80 μmol/L concentration) for 72 h, cells were processed for labeling with fluorescein-tagged dUTP nucleotide and propidium iodide by use of an APO-DIRECT apoptosis kit (Phoenix Flow Systems). The labeled cells were then analyzed by flow cytometry.

*DNA cell cycle analysis.* The cells (60% confluent) were starved for 12 h to arrest them in the $G_0$ phase of the cell cycle, after which they were treated with lupeol (40-80 μmol/L) in RPMI 1640 complete medium for 72 h. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μL cold PBS and 450 μL cold methanol for 1 h at 4°C. The cells were centrifuged at $110 \times g$ for 5 min, and the pellet was washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) at 37°C for 30 min. The cells were chilled over ice for 10 min and stained with propidium iodide (50 μg/mL final concentration) for 1 h and analyzed by flow cytometry.

*Tumorigenicity studies in athymic nude mice.* For these studies, we used 451Lu cells because they develop rapid tumors in the host. A total of $1 \times 10^6$ cells suspended in 50 μL of medium and 50 μL of Matrigel (BD Biosciences) were inoculated s.c. into the right flank of 14 athymic nude mice, 6 weeks old, by using a 27-gauge needle; the mice were divided into two groups. One group of animals received an i.p. injection of lupeol (1 mg/animal) in 0.2 mL of corn oil, whereas the second group received corn oil alone and served as the control group. These treatments were repeated thrice a week on Mondays, Wednesdays, and Fridays. Every week, tumor growth was determined in terms of
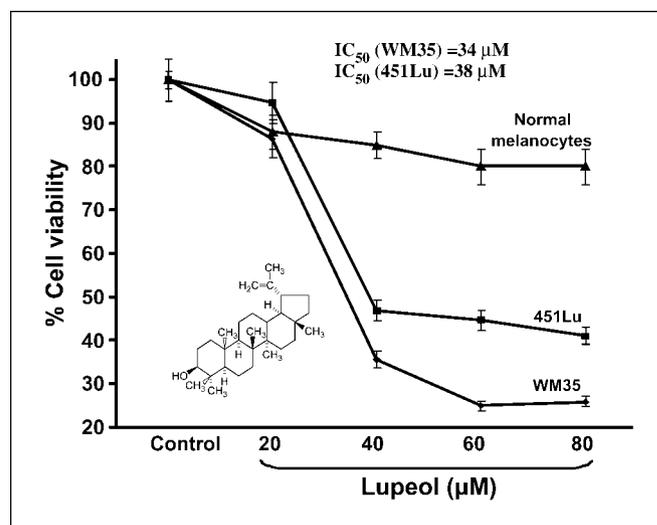


**Fig. 1.** Effect of lupeol on viability of NHMs, nonmetastatic melanoma cells (WM35), and metastatic melanoma cells (451Lu). Cells were treated with specified concentrations of lupeol for 72 h, and cell viability was determined by the MTT assay. Each concentration of lupeol (20-80 μmol/L) was repeated in 10 wells. The values are represented as percent viable cells where vehicle (alcohol + DMSO)–treated cells were regarded as 100% viable. Points, mean value of percent viable cells of three independent experiments; bars, SE. The details are described in Materials and Methods. The structure of lupeol [Lup-20(29)-en-3β-ol] is indicated within the figure.
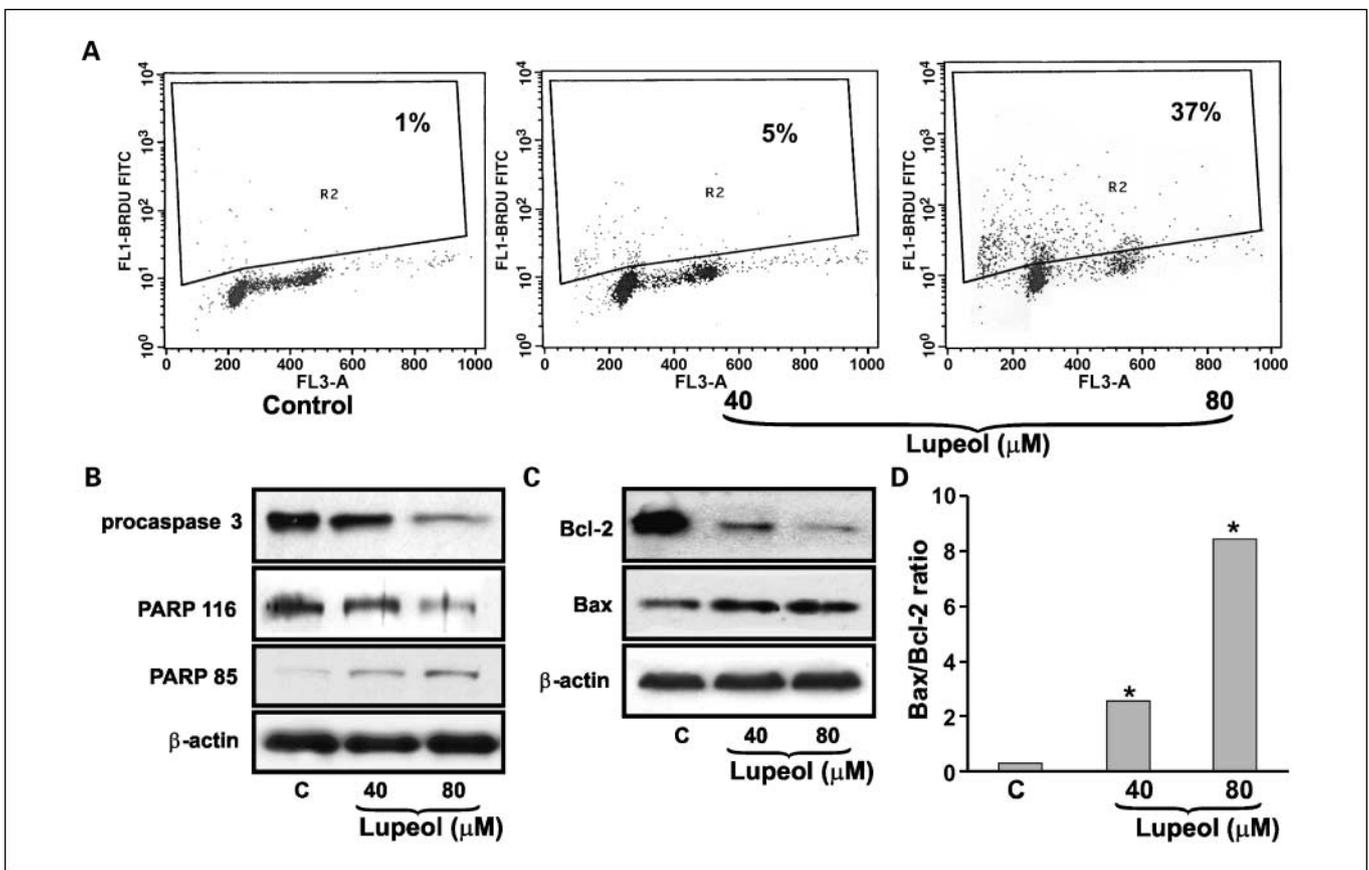
**Fig. 2.** Lupeol induces apoptosis in 451Lu cells as assessed by flow cytometry (*A*) and immunoblot analysis (*B-D*). *A*, quantitative estimation of lupeol-induced apoptosis in 451Lu cells as assessed by flow cytometry. Cells were treated with lupeol (40-80 μmol/L for 72 h), labeled with dUTP using terminal deoxynucleotide transferase and propidium iodide by using an Apo-direct apoptosis kit. Cells showing dUTP fluorescence above that of control population, as indicated by the line in each histogram, are considered as apoptotic cells. The values shown inside each graph indicates percent apoptotic cells. The images are representative of three independent experiments with similar results. The details are described in Materials and Methods. Effect of lupeol treatment on the (*B*) PARP cleavage and protein expression of pro – caspases-3 and (*C*) protein expression of Bcl-2/Bax as determined by immunoblot analysis. *D*, Bax/Bcl-2 ratio. Cells were treated with vehicle (alcohol + DMSO) only or specified concentrations of lupeol for 72 h and harvested; total cell lysates were prepared. PARP cleavage and the expression of Bcl-2, Bax, and pro – caspases-3 were determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. The immunoblots shown here are representative of three independent experiments with similar results. The details are described in Materials and Methods. C, control, represents vehicle (alcohol + DMSO) – treated cells.

volume of tumors (mm³) as a function of time (in days). At the termination of the experiment, animals were sacrificed and tumor tissues and visceral organs were harvested. From the harvested tissues, lysates were prepared and paraffin tumor sections were prepared on slides. The lysates (to be used for biochemical analysis) were stored at -80°C, and paraffin tumor sections were immediately processed for immunohistochemical analysis.

*Immunohistochemical analysis.* Immunohistochemical staining was done as described earlier (14). A pretreatment of sections (to be evaluated for Bcl-2, Ki-67, and cyclin D1) with citrate buffer (pH 6) for 10 min in a microwave was given for antigen retrieval. After antigen retrieval, thick-paraffin-embedded sections (5 μm) were dewaxed, rehydrated, and endogenous peroxidase activity was blocked. Sections were washed in water and PBS and were blocked in blocking buffer (2% goat serum/5% bovine serum albumin in PBS) for 30 min followed by incubation with primary antibody of proliferating cell nuclear antigen (PCNA), Ki-67, Bcl-2, and cyclin D1, at the dilution of 1:50 for 12 h at 4°C. A negative control was included, in which sections were incubated with normal mouse IgG1 replacing the primary monoclonal antibody. After incubation in the primary antibody, sections were washed twice in PBS to remove unbound antibody, followed by incubation for 2 h at room temperature with appropriate horseradish peroxidase – conjugated secondary antibody. Immunoreactive complexes were detected using 3,3′-diaminobenzidine (Dako

Corp.). Slides were then counterstained in hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount. Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer.

*Statistical analyses.* Student's *t* test for independent analysis was applied to evaluate differences between the treated and untreated cells with respect to the expression of various proteins. A Kaplan-Meier survival analysis with the corresponding log-rank analysis and a linear regression analysis was used to measure the rate of mean tumor volume growth as a function of time using S-plus Software (Insightful). A *P* value of <0.05 was considered to be statistically significant.

## Results

*Effect of lupeol on cell viability of NHM, WM35, and 451Lu cells.* Advanced stages of melanoma are mostly resistant to conventional therapeutic agents and only a small percentage of patients respond to the treatment. We investigated the effect of lupeol treatment on the viability of NHM and WM35 and 451Lu cells. The treatment of NHM cells with lupeol (20-80 μmol/L) for 72 h was observed to exhibit only a marginal effect on cell viability (Fig. 1). However, treatment of WM35 and 451Lu cells at similar doses of lupeol significantly

decreased cell viability in a dose-dependent manner (Fig. 1). The $IC_{50}$ for lupeol was estimated to be 32 and 38 μmol/L for WM35 and 451Lu cells, respectively. Based on these observations, we selected a dose of 40 to 80 μmol/L and a period of 72 hours post–lupeol treatment for further mechanistic studies. Because 451Lu represent the highly aggressive and metastatic stage of human melanoma, we selected this cell line for detailed *in vitro* and *in vivo* studies.

*Lupeol induces apoptosis in 451Lu cells.* The inability of tumor cells to undergo apoptosis in response to chemotherapy poses a selective advantage for tumor progression, metastasis, and resistance to therapy in melanoma. We next determined whether lupeol-mediated inhibition of cell viability in 451Lu cells is a result of the induction of apoptosis. We quantified the extent of apoptosis by flow cytometric analysis of lupeol-treated cells. As shown by the data in Fig. 2A, compared with control, lupeol treatment resulted in 5% and 37% TUNEL-positive cells at 40 and 80 μmol/L, respectively, suggesting that treatment of 451Lu cells with lupeol resulted in a dose-dependent induction of apoptosis. Several proteins, including poly(ADP)ribose polymerase cleavage (PARP), play an important role in the condensation and degradation of chromatin of the cells going through the apoptotic death (15). Therefore, the cleavage of

PARP protein is considered as an important biomarker of apoptosis. Next, we measured the cleavage of PARP protein and the expression of procaspase-3 protein in 451Lu cells pretreated with lupeol. Lupeol treatment of cells caused a significant degradation of PARP protein in cells in a dose-dependent manner (Fig. 2B). Similarly, a significant amount of the cleaved product (detected as PARP85) was found in cells treated with lupeol (Fig. 2B). Caspases play an important role as mediators of apoptotic signals from upstream molecules and their activation is considered as a hallmark of apoptosis (16). Next, we measured the expression of procaspase-3 protein. The expression of procaspase-3 protein was observed to be significantly diminished in lupeol-treated cells, suggesting the activation of caspase-3 (Fig. 2B). These data suggest that lupeol induces apoptosis of 451Lu cells by inducing PARP cleavage and caspase-3 activation.

*Effect of lupeol on the expression of mitochondria-dependent apoptotic proteins.* Bcl-2 protein plays a major role in preventing apoptosis and has been linked to chemotherapy resistance in melanoma (17). Prior studies have shown that the expression level of Bax (a proapoptotic protein known to antagonize the anti–Bcl-2) is not significantly decreased in melanoma patients; however, its proapoptotic effects are
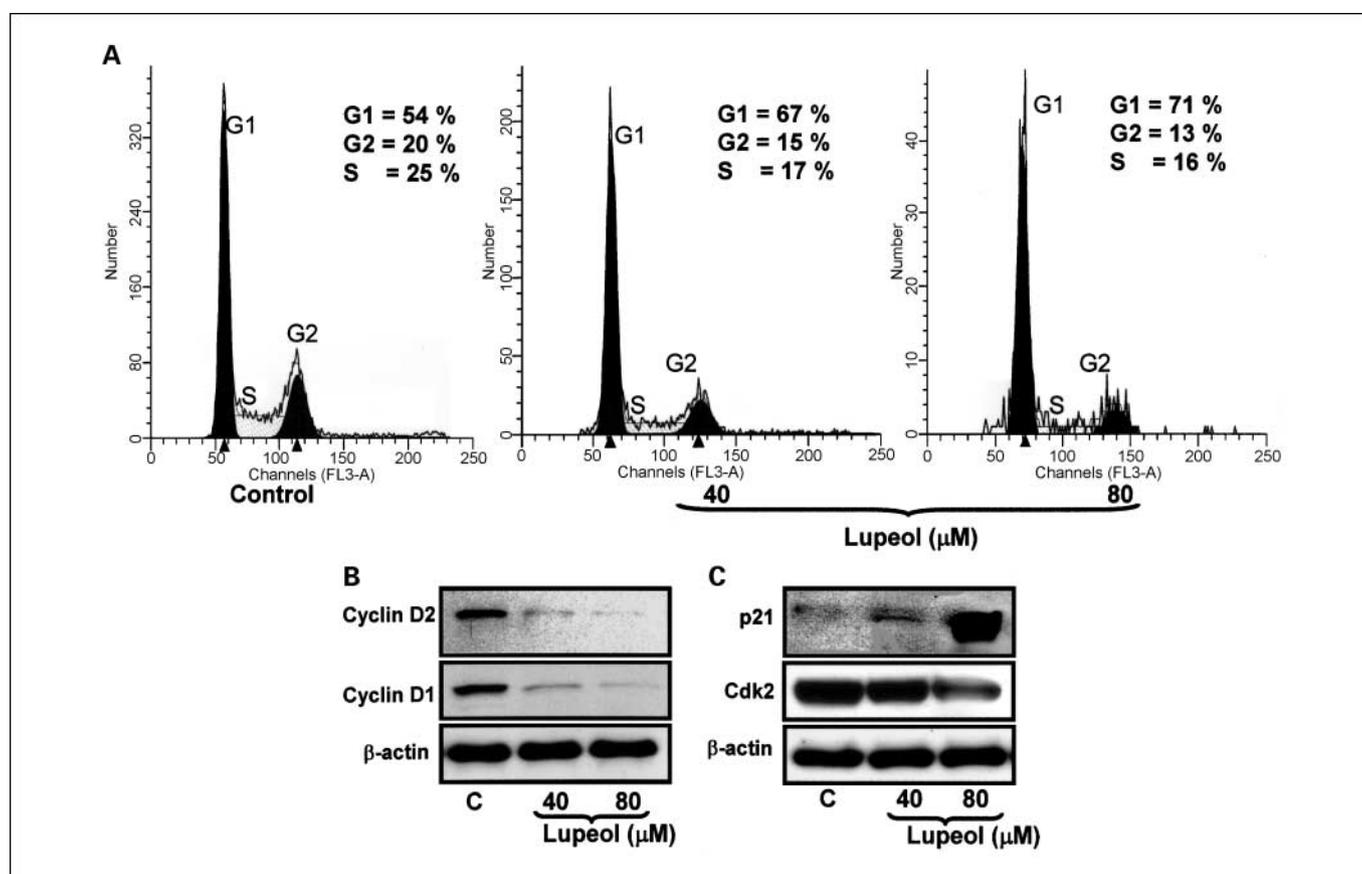
Fig. 3. Effect of lupeol on DNA cell cycle analysis and on the expression of cell cycle regulatory molecules in 451Lu cells as assessed by (*A*) flow cytometry and (*B* and *C*) immunoblot analysis. *A*, the cells were synchronized in $G_0$ phase by depleting the serum for 12 h (*Control*). After 12 h of serum starvation, cells were treated with vehicle or lupeol (40 and 80 μmol/L for 72 h) in complete serum-containing medium and were analyzed by flow cytometry. The percentages of cells in the $G_0$-$G_1$, S, and $G_2$-M phases were calculated using the Cellfit software. Other details are described in Materials and Methods. Data are from a representative experiment repeated thrice with similar results. *B* and *C*, the cells were treated with vehicle only or specified concentrations of lupeol for 72 h and then harvested. The protein expression of (*B*) cyclins D1 and D2, and (*C*) cdk2 and WAF1/p21 were determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. The immunoblots shown here are representative of three independent experiments with similar results. The details are described in Materials and Methods. C, control, represents vehicle-treated cells.

**Fig. 4.** Effect of lupeol administration on the tumorigenicity of 451Lu cells and the expression level of known proliferation markers Ki-67 and PCNA under *in vivo* conditions. A total of $0.5 \times 10^6$ 451Lu cells were suspended in 50 μL of medium and 50 μL of Matrigel. For s.c. tumor growth, the cell suspension was inoculated s.c. into the right flank on 6-wk-old male athymic mice by using a 27-gauge needle. One group of animals received an i.p. injection of lupeol (40 mg/kg body weight) in corn oil whereas animals receiving corn oil alone served as control. Tumors from three animals from control and treated group were excised at 28 d posttreatment when 100% control (corn oil treated) animals attained the tumor volume of ~1,300 mm$^3$. The rest of the animals in the treated group remained on the protocol until they attained a tumor volume of 1,300 mm$^3$. Tumor sections (for immunohistochemistry) and whole-cell lysates (for immunoblot analysis) were prepared from excised tumors. *A,* the graphical representation of data showing the effect of lupeol treatment on growth of tumors from 451Lu cells implanted in athymic nude mice. The growth was measured in terms of average volume of tumors as a function of time. Points, mean; bars, SD. *, $P < 0.05$ from the control group. *B,* the graphical representation of data depicting the number of mice remaining with tumor volumes <1,300 mm$^3$ after treatment with corn oil alone or lupeol for the indicated weeks. *C,* representative photomicrographs (magnification, ×200) showing immunohistochemical staining for Ki-67 and PCNA in tumor sections of corn oil – treated and lupeol-treated mice harvested at 28 d posttreatment. Arrows, regions exhibiting immunoreactivity for Ki-67 and PCNA proteins. The immunostaining data were confirmed in all specimens from each group.
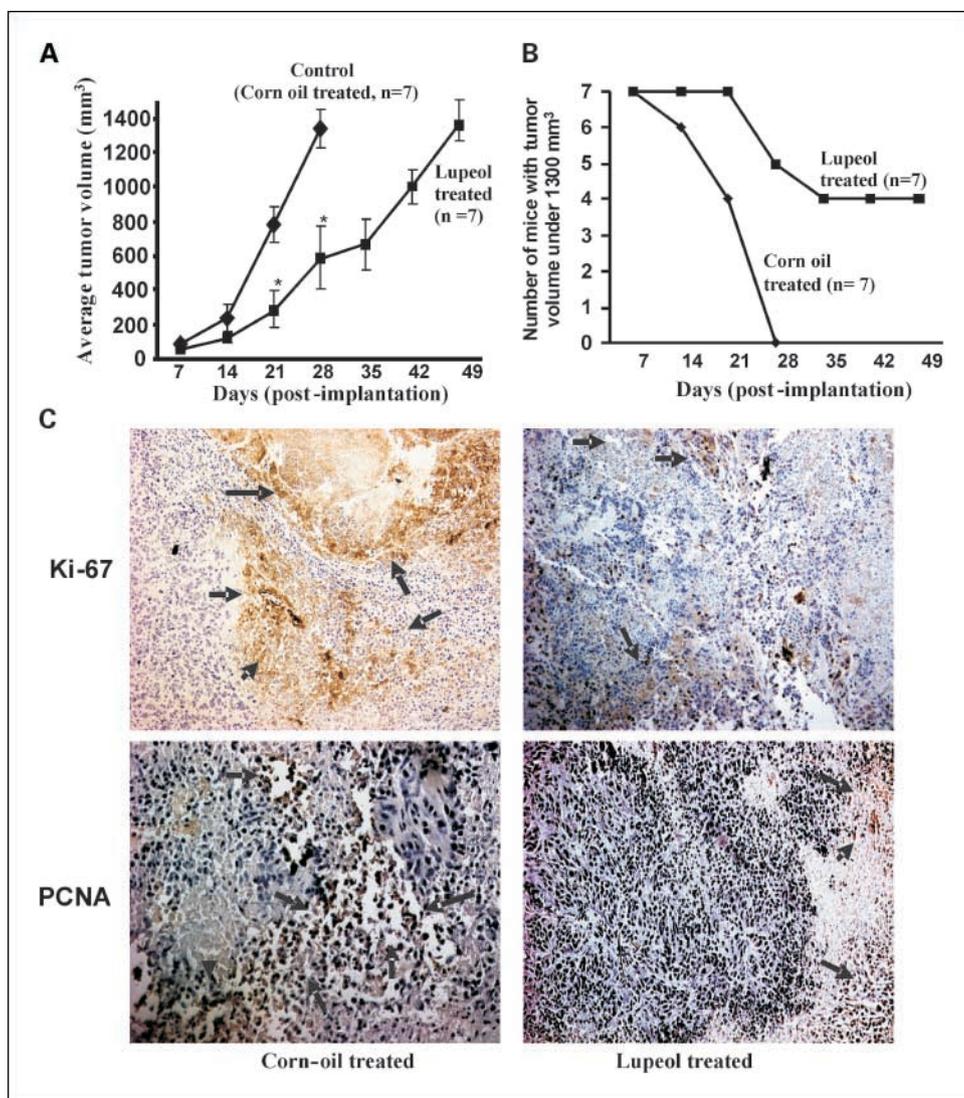
overridden by antiapoptotic Bcl-2 family regulators (17, 18). We next evaluated the effect of lupeol treatment on the expression level of Bcl-2 and Bax proteins. As shown in Fig. 2C, the treatment of cells with lupeol caused a significant decrease in the expression level of Bcl-2 protein and modestly increased the expression level of the Bax protein (Fig. 2C). We next measured the Bax/Bcl2 ratio by comparing the relative densities of the bands depicting the expression level of these proteins. Lupeol treatment was observed to cause a significant shift of Bax/Bcl-2 ratio toward apoptosis in 451Lu cell (Fig. 2D).

*Lupeol induces $G_1$ cell cycle arrest and alterations in $G_1$ cell cycle regulatory proteins in 451Lu cells.* Next, we considered the possibility that inhibition in the growth of 451Lu cells may involve an arrest of cells at specific check point(s) in the cell cycle followed by apoptosis. We therefore assessed the effect of lupeol on cell cycle perturbations. The cells were synchronized by serum deprivation for 12 hours and later incubated with complete medium containing 10% FBS with varying concentrations of lupeol for 72 hours. As shown in Fig. 3A, lupeol treatment resulted in a significant arrest of cells in the $G_0$-$G_1$ phase of cell cycle at 72 hours posttreatment. As shown in

Fig. 3A, lupeol treatment resulted in a significant increase (31-39%) in the number of cells in the $G_1$ phase with a concomitant decrease in the number of cells in the S and $G_2$ phases of the cell cycle at 72 hours posttreatment (Fig. 3A).

Next, we investigated the effect of lupeol treatment on the protein expression of the cyclin/cdk complex, which is operative in the $G_1$ phase of cell cycle. Treatment of 451Lu cells with lupeol resulted in a dose-dependent decrease in the protein expression of cyclin D1 and cyclin D2 (Fig. 3B). The effect of lupeol was also observed on the protein level of cdk2. Treatment of 451Lu cells with lupeol resulted in a dose-dependent decrease in cdk2. The decrease in cdk2 protein expression was more pronounced at 80 μmol/L concentration of lupeol for 72 hours (Fig. 3C). We next assessed the effect of lupeol treatment on the induction of WAF1/p21, which is known to regulate the entry of cells at the $G_1$-S transition checkpoint. Lupeol treatment was observed to result in a significant induction in the expression level of WAF/p21 protein at 80 μmol/L concentration (Fig. 3C).

*Effect of lupeol on tumorigenicity of 451Lu cells in an athymic nude mouse model.* Because lupeol was observed to be effective in inhibiting the growth of metastatic melanoma cells *in vitro*,

we next investigated whether these results could be translated into an *in vivo* xenograft model. Lupeol treatment did not cause any loss in the body weight, food intake, or exhibited apparent signs of toxicity in animals. Implantation of 451Lu cells onto nude mice produced visible tumors in mice with a mean latent period of 14 days. The average volume of tumors in control mice increased as a function of time and reached a preset end point of 1,300 mm$^3$ in 23 days postinoculation. However, at this time, the average tumor volume was only 550 mm$^3$ in mice treated with lupeol (Fig. 4A). Tumors were found to grow an average of 41 mm$^3$ per day in the control group and 17 mm$^3$ per day in the lupeol-treated group. Next, we evaluated whether treatment of lupeol to animals caused a delay in the growth of tumors in nude mice. The observed differences for tumor development in lupeol-treated mice compared with control mice were statistically significant with $P < 0.01$ (Fig. 4B). Approximately 50% of mice that received lupeol treatment did not attain the tumor volume of 1,300 mm$^3$ even at the end of the 7th week (Fig. 4B). Tumors from three animals from control and treated groups were excised at 28 days posttreatment when 100% control (corn oil treated) animals attained the tumor volume of ~ 1,300 mm$^3$. The rest of the animals in the treated group remained on the protocol until they attained a tumor volume of 1,300 mm$^3$. Further, excised tumors (from both groups of animals) exhibited a remarkable difference in their size as observed by the naked eye (data not shown). From these data, we conclude that lupeol is an effective agent that has the potential to inhibit the tumorigenicity of melanoma cells *in vivo*.

*Lupeol administration inhibits the proliferation of 451Lu cell–originated tumors in athymic nude mice.* As lupeol treatment was observed to inhibit or decrease the tumorigenic potential of 45I Lu cells *in vivo*, we next investigated the effect of lupeol administration on the expression level of Ki-67 and PCNA proteins, which are known markers of proliferation in tumor (excised from both groups of animals). It was evident from the immunohistochemical analysis of tumors that animals receiving lupeol administration exhibit significantly decreased Ki-67– and PCNA-positive cells compared with corn oil–treated animals (which exhibited intense staining for these proteins), suggesting the antiproliferative efficacy of lupeol under *in vivo* conditions (Fig. 4C).

Further, cyclins and its regulatory proteins play an important role in the proliferation of melanoma tumor cells (19). Because lupeol treatment was observed to modulate the expression level of cyclin/cdk complex and its regulatory protein p21 under *in vitro* conditions, we next determined the effect of lupeol administration on the expression levels of cyclin D1, cyclin D2, cdk2, and p21 in tumors of both groups of animals. As shown in Fig. 5, lupeol administration was observed to decrease the expression level of cyclin D1, cyclin D2, and Cdk2 proteins (Fig. 5A). A significant increase was also observed in the expression level of p21 protein in tumor tissues of animals treated with lupeol (Fig. 5A). Next, we assessed the expression level of cyclin D1 in the tumor tissues by immunohistochemical analysis. Tumor sections from lupeol administered mice exhibited significantly reduced cyclin D1–positive cells compared with corn oil–treated animal, which exhibit more intense staining for cyclin D1 (Fig. 5B). These data suggest that lupeol administration caused the arrest of growth of melanoma tumors under *in vivo* conditions by interfering in the cyclin/cdk2/p21 complex activity.

*Lupeol administration induces apoptosis of tumor cells in athymic nude mice.* Because lupeol administration was observed to decrease the tumorigenicity of metastatic melanoma cells under *in vivo* conditions, we next asked whether this effect is the end result of growth arrest followed by apoptosis of tumor cells. Next, we investigated the effect of lupeol administration on apoptotic markers (PARP cleavage and activation of caspase-3) in tumor tissues of both groups of animals. As shown in Fig. 5C, lupeol administration was observed to induce the expression level of PARP85 (the cleaved product) and decreased the expression level of PARP116, the complete protein (Fig. 5C). Because Bcl-2 (antiapoptotic protein) and Bax (the proapoptotic protein) were observed to be modulated by lupeol treatment under *in vitro* conditions, we next determined the effect of lupeol administration on the expression level of these proteins in tumor tissues and calculated their ratio. Lupeol administration was observed to significantly decrease the expression level of Bcl-2 protein and a marginal increase in expression level of Bax protein (Fig. 5D). However, when the ratio of the relative densities of both proteins was calculated, a significant shift toward apoptosis was observed in the tumor tissues of lupeol administered animals compared with corn oil–treated animals, which does not exhibit any change in the Bax/Bcl-2 ratio (Fig. 5E). In addition, we determined the expression level of Bcl-2 protein by using immunohistochemical analysis in tumor sections of both groups of animals. In corn oil–treated animals, the Bcl-2–positive staining was significantly higher than in tumor tissues of animals receiving lupeol administration (Fig. 5F). Collectively, these data suggest that lupeol administration induces cell cycle arrest and apoptosis, leading to the reduced tumorigenicity and proliferation of metastatic melanoma tumor cells under *in vivo* conditions.

## Discussion

The prognosis of melanoma patients with distant metastases not amenable to surgery remains poor and is uninfluenced by any treatment intervention yet applied in large randomized multicenter studies (20, 21). One of the major hurdles in the treatment of melanoma is the induction of tumor cell resistance to apoptosis. Because restoring apoptosis has been suggested as a possible therapeutic strategy, a great deal of research has been devoted to understanding the abnormalities in the cellular machinery that causes resistance to apoptosis in melanoma cells (22, 23). Therefore, pharmacologic agents and therapeutic strategies interfering with disrupted apoptosis regulation could improve the therapeutic arsenal against melanoma in the future. With the advent of chemopreventive approaches for the treatment of cancer, there is widespread interest in the possibility that this approach may eventually have an effect on, and could improve the quality of life of, human melanoma patients. Several natural agents with high anticancer efficacy and no or acceptable toxicity to normal tissues are suggested as possible candidates for use by melanoma patients (3, 5–7, 24). In the current study, we provide evidence that lupeol, a diet-based agent, could ameliorate the inefficiency of melanoma cells to undergo apoptosis and inhibit the human melanoma growth *in vitro* and *in vivo*. A striking observation from our data was that both nonmetastatic (WM35) and highly metastatic (451Lu) cells were highly sensitive to lupeol-mediated
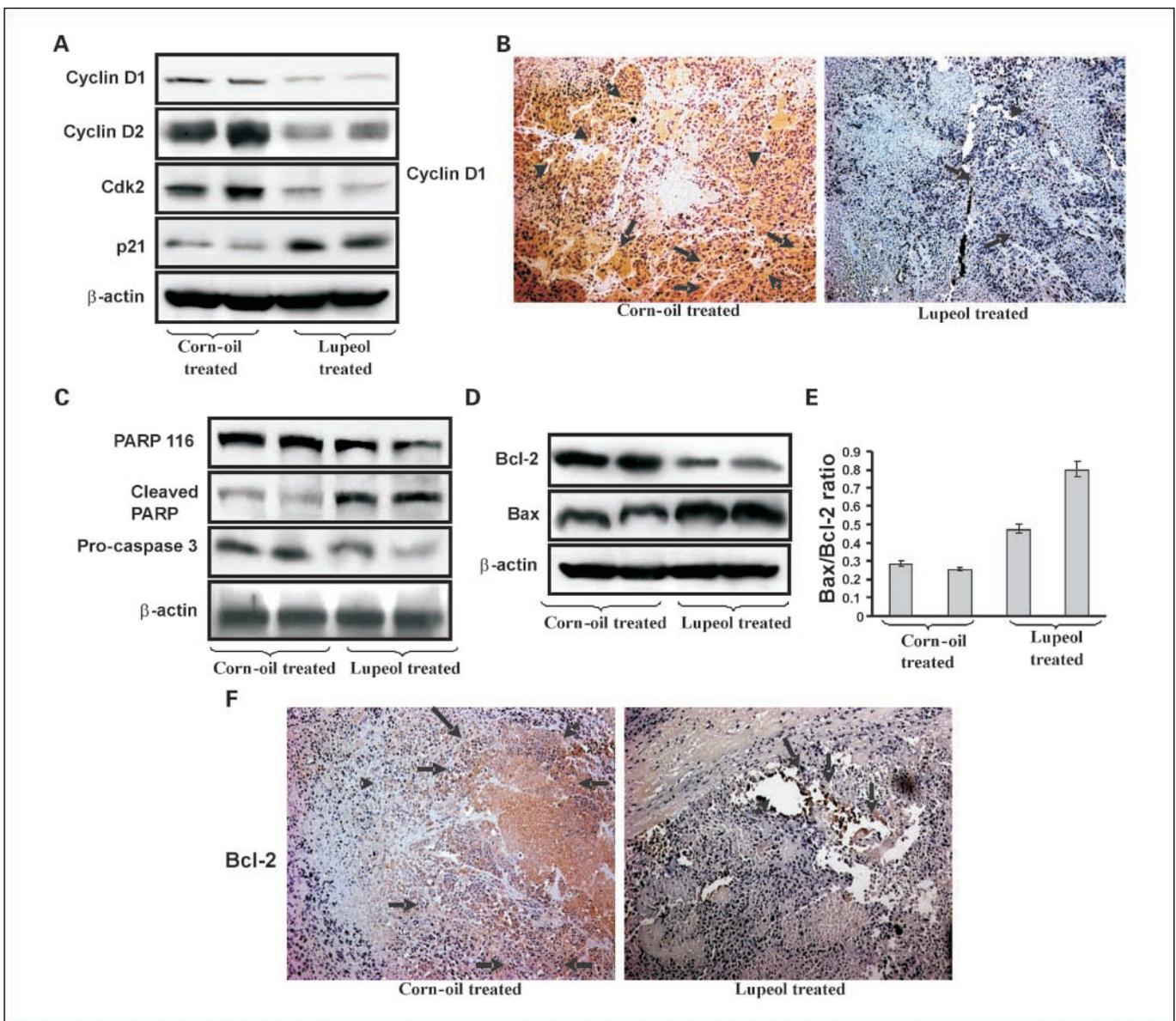
**Fig. 5.** Effect of lupeol administration on the expression level of molecules involved in cell cycle regulation and apoptosis of 451Lu cells under *in vivo* conditions. *A,* effect of lupeol treatment on the expression level of cyclin D1, cyclin D2, cdk2, and p21 in 451Lu tumors excised at 28 d posttreatment were determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. The immunoblot data shown here are representative of two samples from each group. *B,* representative photomicrographs (magnification, ×200) showing immunohistochemical staining for cyclin D1 in tumor sections of corn oil – treated and lupeol-treated mice. Arrows, regions exhibiting immunoreactivity for the cyclin D1 protein. The immunostaining data were confirmed in two or more specimens of each group. *C* and *D,* effect of lupeol treatment on PARP cleavage and on the expression level of pro – caspase-3, Bcl2, and Bax proteins in 451Lu tumors excised at 28 d posttreatment were determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. The immunoblot data shown here are representative of two samples from each group. *E,* representative histogram showing Bax/Bcl-2 ratio in 451Lu cell – propagated tumors. *F,* representative photomicrographs (magnification, ×200) showing immunohistochemical staining for Bcl-2 in tumor sections of corn oil – treated and lupeol-treated mice. Arrows, regions exhibiting immunoreactivity for Bcl2 protein. The immunostaining data were confirmed in all specimens of each group. The details for immunohistochemical analysis are described in Materials and Methods.

loss of viability, and only a marginal effect was observed in normal melanocytes at similar doses. Our data are significant because in recent years, the emphasis is on natural diet-based agents capable of selective/preferential elimination of cancer cells by inhibiting cell cycle progression and/or causing apoptosis while sparing normal cells. In addition, these data provide evidence that lupeol has the potential to kill the tumor cells of advanced-stage characteristics such as 451Lu cells, which are generally resistant to conventional chemotherapeutic agents.

Studies have shown the strong immunoreactivity of Bcl-2 protein in cells of human and murine melanocytic origin, and experimental evidence suggests a role for Bcl-2 in the inhibition of programmed cell death in human and murine malignant melanoma cells (25). Recent clinical studies have shown the promise of Bcl-2 antisense therapy in patients with melanoma (26). In current study, we observed that lupeol treatment causes a significant reduction in the expression level of Bcl-2 protein in metastatic melanoma cells under cell culture conditions as well as in melanoma tumor tissues of animals receiving lupeol

administration. These data provide compelling evidence that lupeol has the potential to cause apoptotic death of metastatic melanoma cells by modulating Bcl-2 protein expression. Considerably less information is known about Bax protein (the negative regulator of Bcl-2) in human melanoma (18). In the current study, lupeol treatment was observed to significantly increase the expression level of Bax protein in metastatic melanoma cells. Further, a modest increase in the expression level of Bax was also observed in melanoma tumor tissues of animals receiving lupeol treatment. Collectively, these data suggest that lupeol has a potential to cause a shift in the Bax/Bcl2 ratio toward apoptosis, resulting in the inhibition/or reduction in the tumorigenicity of metastatic melanoma cells.

Of the many regulatory checkpoints of the cell cycle, the acquisition of abnormalities at the $G_1$-S checkpoint seems to be the most crucial step in the genesis and progression of melanoma (2, 19). In present study, we observed that lupeol treatment caused a cell cycle arrest of metastatic melanoma cell at the $G_1$-S phase. Interestingly, lupeol was also found to reduce the proliferation of metastatic melanoma cells under *in vivo* conditions as is evident from the data that shows a significant decrease in the staining pattern of proliferation markers such as PCNA and Ki-67 in melanoma tumor tissues. Expression of cyclin D1 has been shown at constitutively high levels in melanoma cell lines and metastatic tissues, suggesting its oncogenic role in melanoma pathogenesis (2, 19, 27).

The importance of cyclin D1 as therapeutic target for melanoma emanates from the studies showing that antisense treatment causes apoptosis and tumor shrinkage of tumors in mice implanted with cyclin D1–overexpressing melanoma xenografts (28). It is noteworthy that lupeol treatment significantly decreased the expression level of cyclin D1 protein in metastatic melanoma cells as well as in tumor tissues of mice. Further, the number of cyclin D1–positive stained cells were significantly lesser in tumor tissues of lupeol-treated mice compared with control. These data provide convincing evidence about the efficacy of lupeol as a potent inhibitor of cyclin D1. Our data is significant because earlier reports suggested that the cyclin D1 protein could be a target for therapeutic intervention of melanoma. The p21 protein is known to mediate the growth suppression effects by arresting the cell cycle at the $G_1$-S checkpoint. Although a few reports have shown an increased p21 protein expression in melanoma, the majority of studies have shown that the expression of p21

protein is lower in the metastases compared with the corresponding primary melanoma lesions, suggesting that downregulation of p21 expression is associated with development of a metastatic phenotype (29, 30). In the current study, we observed that lupeol treatment caused a significant increase in the expression level of p21 protein in metastatic melanoma cells *in vitro* and metastatic melanoma tumors *in vivo*. Because cyclins in association with cdks drive the cell cycle and the observation that lupeol decreases the expression level of cdk2, the modulation of cyclin D1/cdk2/p21 complex could be a possible explanation for the observed cell cycle arrest melanoma cells by lupeol (Fig. 3). Taken together, these data suggest that lupeol, by targeting Bcl2/Bax and cyclin D1/cdk2/p21 complexes, induces $G_1$-S phase cell cycle arrest, which is followed by apoptosis of metastatic melanoma cells.

Because lupeol treatment significantly inhibited the growth of metastatic melanoma cells under *in vitro* conditions, we asked whether these data could be translated under *in vivo* situations. An i.p. administration of lupeol to athymic nude mice showed inhibitory effects against the growth of melanoma cell–derived tumors. These *in vivo* growth-inhibitory effects of lupeol could be explained by the biochemical mechanisms observed in the present study. These data are significant because the athymic nude mouse model is an extremely useful mouse model to study the therapeutic potential of anticancer agents and is relevant to the human situations. In addition, this mouse model has been used to study the effects of lupeol against prostate cancer and head and neck squamous cell carcinoma in recent studies from our laboratory and others (31, 32).

To summarize, our present findings showed the *in vitro* and *in vivo* anticancer efficacy of lupeol, with mechanistic rationale (Bcl2-mediated apoptosis induction/$G_1$-S cell cycle arrest) against metastatic human melanoma cells without any significant cytotoxicity to nonneoplastic melanocytes. These observations warrant further *in vivo* efficacy studies in models that mimic progressive forms of human melanoma as well as estimation of pharmacologically achievable doses having biological significance in *in vitro* studies. The positive outcomes of such an *in vivo* study could form a strong basis for the development of lupeol as a novel agent for human melanoma prevention and/or intervention alone or as an adjuvant to known therapeutic agents such as Oblimersen (an anti-Bcl2 agent) for the treatment of melanoma.

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