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Lupeol induces p53 and cyclin-B-mediated G2/M arrest and targets apoptosis through activation of caspase in mouse skin

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ABSTRACT

Lupeol, present in fruits and medicinal plants, is a biologically active compound that has been shown to have various pharmacological properties in experimental studies. In the present study, we demonstrated the modulatory effect of lupeol on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced alterations on cell proliferation in the skin of Swiss albino mice. Lupeol treatment showed significant ($p < 0.05$) preventive effects with marked inhibition at 48, 72, and 96 h against DMBA-mediated neoplastic events. Cell-cycle analysis showed that lupeol-induced G2/M-phase arrest (16–37%) until 72 h, and these inhibitory effects were mediated through inhibition of the *cyclin-B*-regulated signaling pathway involving *p53, p21/WAF1, cdc25C, cdc2*, and *cyclin-B* gene expression. Further lupeol-induced apoptosis was observed, as shown by an increased sub-G1 peak (28%) at 96 h, with upregulation of *bax* and *caspase-3* genes and downregulation of anti-apoptotic *bcl-2* and *survivin* genes. Thus, our results indicate that lupeol has novel anti-proliferative and apoptotic potential that may be helpful in designing strategies to fight skin cancer.

Introduction

Human skin forms a barrier between the host and the physical, chemical, and biological environments [1,2]. However, it is also a potential portal of entry for hazardous agents and a potential target of environmental toxins such as industrial chemicals, arsenic, pesticides, cigarette smoke, and pollutants, which can result in skin-related health problems such as skin cancer [3]. It has been estimated that almost one third of all new cancers diagnosed annually worldwide originates in the skin [4]. The increased incidence of skin cancer has led to increased interest in the processes by which these tumors develop and how they can be prevented. Strategies that focus solely on protecting against the effects of UV radiation and chemical carcinogens have, at best, had a modest impact on the prevention of skin cancers caused by environmental stress. Therefore, newer chemopreventive methods are necessary to protect the skin from the damaging effects of both chemical agents and solar UV radiation [5]. The concept of chemoprevention is based on identifying early biologic events that occur in the epithelium and targeting these events to prevent progression to malignancy [6]. In recent years, the use of phytochemicals that are present in the diet and beverages consumed by humans have gained considerable attention as chemopreventive agents that can slow, reverse or prevent the process of carcinogenesis [7].

Lupeol (Lup-20(29)-en-3-β-ol), a naturally occurring pentacyclic triterpene, is a principal constituent of fruit plants such as olive, mango, fig, and medicinal herbs that have been used to treat skin ailments [8]. Lupeol has been shown to have analgesic, anti-inflammatory [9], antioxidant [10], and cytoprotective properties [11], along with antimutagenic [12] and hypotensive activities [13]. Several authors have reported that lupeol exerts antitumor effects by triggering apoptosis in cancer cells without affecting normal cells [14,15]. Lupeol also has antitumor effects in the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mouse skin tumorigenesis model through the inhibition of TPA-induced activation of Phosphoinositide-3-kinase (PI3K), activation of Nuclear factor κB (NF-κB) and Inhibitor κB Kinaseα (IKKα), and degradation and phosphorylation of Inhibitor κBα (IκBα) [16]. Extending the previous studies on chemopreventive properties of lupeol, we examined the short-term anti-proliferative and apoptotic potentials of lupeol on DMBA-induced carcinogenic events in mouse skin. We also hypothesize that cell-growth inhibition by lupeol can be attributed to cell-cycle regulation and apoptosis. These studies indicate that lupeol has beneficial effects and potential to be developed as a potent nontoxic agent against skin cancer.

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; PI, propidium iodide; RT-PCR, reverse transcriptase polymerase chain reaction; MPF, M-phase-promoting factor; IAPs, inhibitors of apoptosis proteins; PBS, phosphate buffer saline.

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Materials and methods

Materials. Lupeol, DMBA, ribonuclease A, and propidium iodide (PI) were obtained from Sigma (St. Louis, USA). All other chemicals used were of analytical grade purity and procured locally.

Animal bioassay. Swiss albino mice (Female, 20–22 g body weight) were obtained from the Indian Institute of Toxicology Research (Lucknow, India) animal-breeding colony. Ethical approval for the experiment was obtained from the institutional ethics committee. The animals were caged in polypropylene cages and housed ten animals per cage on wood-chip bedding in an air-conditioned (temperature 23 ± 2 °C, relative humidity 55 ± 5%) animal room. Animals were quarantined for 1 week and subject to a 12-h light/dark cycle and were fed a solid pellet diet (Ashirwad, Chandigarh, India) and water *ad libitum*. The animals were divided into 5 groups, each consisting of 20 animals. Lupeol (1 mg/mouse) was administered daily and DMBA was applied topically (100 μg/mouse) on the first day in the interscapular region of 2 cm^2. Group I was the control group and animals received no treatment. Group II was used as a positive control group and only received DMBA 100 μg/mouse. Group III animals were administered lupeol 1 mg/mouse 1 h before DMBA treatment. Group IV animals were administered the doses of lupeol as mentioned above 1 h after DMBA treatment. Group V animals received lupeol (1 mg/mouse) alone.

Five animals from each group were euthanized by cervical dislocation at 24, 48, 72 and 96 h after the first treatment, respectively. The skin from the painted area was excised, washed in chilled phosphate-buffered saline (PBS), and the fat layer was removed using a sterilized scalpel blade. The skin tissues were stored at −80 °C until used for further experimentation.

Flow-cytometric analysis. Single-cell suspensions of skin tissue from treated and control groups were prepared using Medimachine (Beckton Dickinson, San Jose, USA) as described previously [17]. For flow-cytometric analysis, cells in suspension were fixed in chilled 70% ethanol. Cells were centrifuged from the fixative and treated with 0.1% Triton X-100 for 5 min. After incubation, cells were again centrifuged and resuspended in 1 ml of PBS, ribonuclease A (100 μg/ml) was added, and the cells were incubated at 37 °C for 30 min. After further centrifugation, cells were resuspended in 1 ml of PBS and 50 μg/ml PI and incubated for 30 min at 4 °C. The data were acquired and analyzed using a flow-cell cytometer (Becton–Dickinson LSR II, San Jose, CA, USA) and 'Cell Quest' software.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the treated and control skin tissue using TRI Reagent (Sigma, St. Louis, USA) according to the manufacturer's instructions. RT-PCR was conducted as described previously [18] with some modifications. In brief, cDNA was prepared using RNA samples (3–5 μg) to which 1 μg oligo$(dT)_{18}$, 0.5 mM dNTP and 200 U of Revert Aid™ H-Minus M-MuLV RT enzyme were added (MBI Fermentas, USA). PCR analysis was performed using selective mouse primers (Table 1) (synthesized at Integrated DNA Technology, Inc., Coralville, IA) and 2 μl of RT product were incubated with 1 U of *Taq* DNA polymerase in a 50-μl reaction mixture containing 1 mM dNTP and 1.5 mM $MgCl_2$ (MBI Fermentas, USA). The amplified fragments were detected in 2% (w/v) agarose gel and analysed using an IS1000 image analysis system (Alpha Innotech, San Leandro, CA, USA). Another set of primers were used for amplification of an endogenous control gene (GAPDH, 583 bases). The amplified fragments were detected by 2% (w/v) agarose gel electrophoresis and ethidium bromide (0.3 μg/ml) staining (Sigma). Each band was analyzed using an IS1000 image analysis system (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. All data are expressed as mean ± SE of 3 independent experiments. Statistical significance was determined using Student's *t*-test and a *p*-value of less than 0.05 was considered statistically significant.

Results and discussion

It has been suggested that intranuclear mechanisms after DNA damage are highly conserved in DNA repair or cell-cycle arrest [19]. Extensive DNA damage leads to prolonged cell-cycle arrest and cell death [20,21]. Meanwhile, cell-cycle arrest due to lupeol treatment, as in the present study, allows cells to repair damaged DNA, complete DNA replication, or both, thereby further inhibiting the carcoinogenesis process. Flow-cytometric analysis of the cell-cycle checkpoint response after DNA damage revealed a complex series of events, with perturbation of the cell cycle. Our results showed an increase in the percentage of cells in S-phase in DMBA-exposed mouse skin (Group II) compared with the control group (Group I) in all time points. In Group V, which only received lupeol, distribution of cells was similar to that in the control group (Group I) (data not shown). However, lupeol-induced accumulation of G2/M-phase cells was observed in Groups III and IV (Fig. 1). Specifically, G2/M block was first detected at 24 h after

Table 1
Mouse-specific primers used in RT-PCR.

Name	Sequence	Conditions for PCR reaction
GAPDH (internal standard)	FP: 5′-CCATCACCATCTTCCAGGAG-3′ RP: 5′-CCTGCTTCACCACCTTCTTG-3′	95 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s.
bax	FP: 5′ AAGCTGAGCGAGTGTCTCCGGCG 3′ RP: 5′ GCCACAAAGATGGTCACTGTCTGCC 3′	95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.
bcl-2	FP: 5′ CTCGTCGCTACCGTCGTGACTTCG 3′ RP: 5′ CAGATGCCGGTTCAGGTACTCAGTC 3′	95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.
cdc2	FP: 5′ GGACTACAAGAACACCTTTC 3′ RP: 5′ ACGGAAGAGAGCCAACGGTA 3′	95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s.
cdc25c	FP: 5′ GTATATGGAGCTGTGTGACC 3′ RP: 5′ CCTCCTTAGTGCTAGGACGA 3′	95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s.
cyclin-B	FP: 5′ GGAAGAAACTGCAGGTGGTC 3′ RP: 5′ TGCTGCTGGCATACTTGTTC 3′	95 °C for 30 s, 57 °C for 30 s, and 72 °C for 60 s.
p21/waf1	FP: 5′ AATCCTGGTGATGTCCGACC 3′ RP: 5′ TTGCAGAAGACCAATCTGCG 3′	94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min.
P53	FP: 5′ ATGACTGCCATGGAGGAGTCACAGT 3′ RP: 5′ GTGGGGGCAGCGTCTCACGACCTCC 3′	95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s. .
survivin	FP: 5′ TGGCAGCTGTACCTCAAGAA 3′ RP: 3′ AGCTGCTCAATTGACTGACG 3′	95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s.
caspase-3	FP: 5′ AGTGACCATGGAGAACAACA 3′ RP: 5′ AGCTGCTCCTTTTGCTATGA 3′	95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min.