Hepatocellular carcinoma (HCC) is the fifth most malignant tumor worldwide and is known to be resistant to conventional chemotherapy. New therapeutic strategies are urgently needed for treating HCC. Lup-20(29)-en-3H-ol (Lupeol), a novel dietary triterpene, is found in fruits, vegetables, and medicinal plants and possesses multiple bio-activities with very low toxicity. In the current study, we investigated its growth-inhibitory effects in HCC cell lines SMMC7721 and HepG2. In the in vitro studies, lupeol treatment alone caused decrease of cell viability in two HCC cell lines in a dose-dependent manner. It also induced apoptosis and caused cell accumulation in S phase. Further analysis revealed the induction of active caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage by lupeol treatment. In the in vivo studies, nude mice implanted with SMMC7721 cells subcutaneously were treated with lupeol three times a week and tumor development was significantly inhibited. We further investigated the combination anti-tumor effect of lupeol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in HCC, considering TRAIL treatment alone could not achieve high level of anti-tumor effect. The results demonstrated that lupeol could exert a combinational effect with TRAIL, resulting in chemosensitization of HCC. Our results suggested that lupeol alone or as an adjuvant to therapeutic agents could be developed as a potential agent for treating HCC.

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and induce apoptosis of HCC cell lines SMMC7721 and HepG2. Lupeol treatment alone significantly suppressed the tumor growth in nude mice. Our results also suggested that lupeol could exert a combinational effect with TRAIL, resulting in chemosensitization of HCC.

MATERIALS AND METHODS

Cell Lines and Culture Human HCC cell line HepG2 and human liver cell line Lo2-9 were gifts from Dr. Limin Zheng (School of Life Sciences, Sun Yat-sen University). Human HCC cell line SMMC7721 was purchased from Cell Bank, Chinese Academy of Sciences. They were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 10 mg/ml penicillin G and 50 μg/ml streptomycin (Shanghai Sangon Biological Engineering Technology & Services Co., Shanghai, China) at 37 °C in a humidified atmosphere containing 5% CO2.

Antibodies and Reagents Antibodies against β-actin, active caspase-3 and cleaved poly(ADP-ribose)polymerase (PARP) were purchased from BD (Franklin Lakes, NJ, U.S.A.). Lupeol (molecular weight (MW) 426.72) was purchased from Sigma-Aldrich (Rocky Hill, NJ, U.S.A.). BCA Protein Assay Kit was purchased from Beyotime (JiangSu, China). A stock solution of lupeol (30 mmol/l) was prepared by resuspension in warm alcohol and dilution in dimethyl sulfoxide (DMSO) at 1 : 1 ratio.

Cell Viability Assay The effect of lupeol on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. The cells were plated at 2.5×103 per well in 100 μl of complete culture medium 24 h before the assay. Cells were treated with lupeol (20—100 μmol/l) for 48 h. The final concentrations of DMSO and alcohol were lower than 0.25%. After incubation for 48 h at 37 °C in a humidified incubator, MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each well and incubated for 4 h; then the medium was removed, 0.1 ml of buffered DMSO was added to each well. The absorbance was recorded on a microplate reader at the wavelength of 490 nm. The effect on cell growth inhibition was assessed as percent cell viability wherein vehicle-treated cells were taken as 100% viable.

Cell Cycle Analysis After lupeol treatment, the DNA content and cell cycle distribution of SMMC7721 and HepG2 cells were determined by flow cytometry. The cells were plated at a density of 2×105 per well and were harvested with or without lupeol treatment at 12, 24 and 48 h. The cells were washed once in PBS. They were then fixed in cold 70% ethanol and stored at 4 °C for 30 min. Then ethanol was removed and the cells were resuspended in PBS. The fixed cells were then washed with PBS, treated with RNase (1 μg/ml), and stained with propidium iodide (50 μg/ml) for 30 min at 37 °C. Cell cycle analysis was performed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, U.S.A.) and analyzed by Flowjo software.

Quantification of Apoptosis For apoptosis analysis, SMMC7721 and HepG2 cells (5×105) were plated in each well of the six-well plate and treated with different doses (0, 25, 40, 50 μmol/l) of lupeol in 10% fetal bovine serum-DMEM for 48 h. The cells were then labeled with Annexin V and 7-Aminoactinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, U.S.A.).

Western Blot Analysis HCC cells (50% confluent) were treated with lupeol (0, 25, 30, 50 μmol/l) for 48 h in complete cell medium. After 48 h of treatment with lupeol, the cells were harvested and cell lysates were prepared and stored at −80 °C for later use. The protein content in the lysates was measured by BCA Protein Assay Kit (Beyotime, JiangSu, China). For Western blot analysis, 25 μg of protein were resolved over 12% tris-glycine polyacrylamide gels under nonreduced conditions, transferred onto polyvinylidene difluoride (PVDF) membranes, and subsequently incubated in blocking buffer (5% nonfat dry milk) for 12 h at 4 °C. The blots were incubated with appropriate primary antibody, washed, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Carpinteria, CA, U.S.A.). The blots were detected with chemiluminescence (ECL-Kit, Beyotime, JiangSu, China) followed by autoradiography. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody.

Animal Studies A total of 2×106 SMMC7721 cells suspended in 0.2 ml PBS were inoculated subcutaneously (s.c.) into the right flank of 30 nude mice using 1-ml needles. A week later, the nude mice were randomly distributed into three groups and each group consisted of 10 nude mice. They were treated with 20 mg/kg or 80 mg/kg lupeol intraperitoneally (i.p.) in 0.2 ml corn oil, with corn oil alone as the control group, three times per week for 30 d. The tumor volumes were measured once a week. The following formula was used for tumor volume measurement: (short diameter)2×long diameter/2. At the end of the experiment, animals were sacrificed and tumor tissues were weighed. The design of the study was approved by the Ethical Committee of Soochow University.

Statistical Analysis All data represents at least three independent experiments and results were shown as mean±S.D. Statistical differences between two groups were determined by Student’s t-test. analysis of variance (ANOVA) analysis was applied for multiple group comparison. A significant difference was considered as p<0.05.

RESULTS

Effect of Lupeol on Cell Viability and Apoptosis of HCC Cell Lines HCC cell lines SMMC7721 and HepG2 were treated with lupeol at different doses (Fig. 1A). Lupeol treatment inhibited the growth of the two HCC cell lines in a dose-dependent manner. There was no significant difference in drug sensitivity (IC50: 45 μmol/l and 48.5 μmol/l) between the two HCC cell lines. To examine its toxicity to normal cells, a human liver cell line, Lo2-9, was also treated with lupeol. The result showed that Lo2-9 was less sensitive to lupeol, with the IC50 up to 90 μmol/l. It suggested that lupeol could effectively and selectively affect the viability of HCC cell lines SMMC7721 and HepG2.

The effect of lupeol on cell cycle distribution was also
evaluated by flow cytometry (Fig. 1B). When lupeol was administered at the dose of IC\(_{50}\) (45 μmol/l), SMMC7721 cells exhibited increased cell percentages in S phase (from 16 to 36%) in a time-dependent manner. HepG2 cells also showed S phase arrest with lupeol treatment (data not shown). To further investigate whether lupeol could induce apoptosis of the HCC cells, the apoptotic cell percentages were analyzed by flow cytometry (Fig. 1C). Lupeol induced apoptosis of SMMC7721 cells in a dose-dependent manner. Compare with the control, lupeol treatment resulted in early apoptosis rates of 5.16%, 21.84% and 36.44% at the doses ranging from 25 to 50 μmol/l. Lupeol could also induce apoptosis of HepG2 cells in a dose dependent manner (data not shown).

To further demonstrate the induction of apoptosis by lupeol at the molecular level, we evaluated PARP cleavage as a measure of apoptosis. As is evident from the immunoblots, lupeol-induced PARP cleavage was highly increased at the dose of >50 μmol/l (Fig. 2). Caspase cascade is also the hallmark of apoptosis. We detected the increased expression level of active-caspase-3 in SMMC7721 and HepG2 cells treated by lupeol.

**Lupeol Suppressed The Tumor Growth in A Murine Model of HCC** In order to determine the tumor suppressive effect of lupeol in vivo, we next examined the effect of lupeol in a murine model of HCC. Lupeol was used at a dose of 20 mg/kg or 80 mg/kg, with corn oil as the control. The treatment began 7 d after the implantation of SMMC7721 cells. There was a significant increase in body weight in the lupeol (80 mg/kg) group (21.32 ± 0.79 g) compared with the control group (19.72 ± 0.53 g) (p < 0.05) (Fig. 3A). Lupeol exhibited no apparent sign of toxicity in animals. We also observed that tumor development was significantly suppressed in lupeol (80 mg/kg)-treated mice comparing with the control group (Fig. 3B). The tumor weights of lupeol (80 mg/kg)-treated mice (62.08 ± 18.50 mg) were less than those of the control group (133.83 ± 28.94 mg) (p < 0.05) (Fig. 3C). The results suggested that lupeol was an effective agent that could inhibit the growth of transplanted HCC tumors in vivo.

**Combinational Effect of Lupeol and rTRAIL on HCC Cell Lines** The MTT assay showed that SMMC7721 and HepG2 cells were highly chemoresistant to rTRAIL therapy (Fig. 4A). The dose of 1000 ng/ml resulted in 33 to 36% inhibition of cell growth in SMMC7721 and HepG2 cells. There was no obvious increase in growth inhibition when the concentration was higher than 1000 ng/ml (data not shown). We investigated whether combinational treatment of lupeol and rTRAIL could increase the growth-inhibition rate of HepG2 comparing with rTRAIL treatment alone (Fig. 4B). Lupeol treatment (25 μmol/l) for 48 h resulted in 7% inhibition of HepG2 cell growth, while rTRAIL treatment of 50 ng/ml or 100 ng/ml alone for 48 h resulted in 18% and
was no data of the growth inhibitory effect of lupeol in vivo significantly decreasing the expression of DR3. However, there inhibit the growth of HCC cell line SMMC7721 by significantly increased the percentages of cells in S phase. This could be due to the S phase arrest or early G2/M phase arrest. In 7,12-dimethylbenz(a)anthracene (DMBA)-induced mouse skin cancer model, lupeol induced G2/M-phase arrest by inhibiting the cyclin-B-regulated signaling pathway involving p53, p21/WAF1, cdc25C, cdc2, and cyclin-B gene expression. Therefore, lupeol could induce early G2/M arrest through the similar mechanisms in HCC cells.

31% inhibition of HepG2 cell growth, respectively. However, when we combined lupeol and rTRAIL treatment, HepG2 cells exhibited 26% and 39% growth inhibition, which were significantly higher than either treatment alone. We further examined the effect of combinational treatment with lupeol and rTRAIL on cell apoptosis. Lupeol and rTRAIL treatment induced HepG2 cell apoptosis at the level significantly higher than either treatment alone (Fig. 4C). Interestingly, the combination treatment with lupeol and rTRAIL specifically increased the percentage of cells in late stage apoptosis or necrosis (44.32%), while lupeol or rTRAIL alone did not (15.75% and 13.68%, respectively). As evident from microscopy analysis, the combined treatment with lupeol and rTRAIL significantly reduced the cell viability compared with lupeol and rTRAIL treatment alone (Fig. 4D). These results suggested that lupeol could potentially be used for combination therapy with rTRAIL for treating HCC patients.

**DISCUSSION**

Lupeol has been shown recently in an *in vitro* system to inhibit the growth of HCC cell line SMMC7721 by significantly decreasing the expression of DR3. However, there was no *in vivo* data of the growth inhibitory effect of lupeol in SMMC7721 cells-derived tumors. Our results suggested lupeol could not only selectively induce apoptosis of HCC cell lines SMMC7721 and HepG2, but also suppress HCC tumor growth *in vivo*. The expression level of the hallmark proteins during apoptosis, including active-caspase-3 and cleaved PARP significantly increased after lupeol treatment. We also observed that lupeol treatment significantly increased the percentages of cells in S phase. This could be due to the S phase arrest or early G2/M phase arrest. In 7,12-dimethylbenz(a)anthracene (DMBA)-induced mouse skin cancer model, lupeol induced G2/M-phase arrest by inhibiting the cyclin-B-regulated signaling pathway involving p53, p21/WAF1, cdc25C, cdc2, and cyclin-B gene expression. Therefore, lupeol could induce early G2/M arrest through the similar mechanisms in HCC cells.

In nude mouse model of HCC, lupeol (80 mg/kg) significantly suppressed the growth of SMMC7721 cells-derived tumors, with no sign of toxicity. Interestingly, the body weights of lupeol treated group were higher than the control group. The possible explanations were that lupeol exhibited no apparent signs of toxicity in animals, as well as suppressed the growth of SMMC7721 cells-derived tumors in nude mice, therefore improved the overall health and increased body weights of the lupeol-treated mice.

It has been shown that TRAIL is a promising agent for treatment of cancer because TRAIL selectively suppresses tumor growth *in vivo* and *in vitro*. However, HCC are not sensitive to soluble rTRAIL treatment. Our data are in agreement with other studies showing HCC cells HepG2 and SMMC7721 were resistant to rTRAIL treatment. The basis of resistance of HCC cells to TRAIL could be due to failure at any step in the death signaling cascade. For example, TRAIL resistance can be located at receptor level, such as down-regulation the expression level of TRAIL receptors (DR4 and DR5), or at death-inducing signaling complex (DISC) level mediated by proteins counteracting DISC formation and by proteins inhibiting caspase-8 activation, due to up-regulation the expression level of cFLIP protein. cFLIP is constitutively expressed in all human HCC cell lines and is expressed more in human HCC tissues than in nontumor liver tissues. Furthermore, it could be also due to an inability to activate mitochondria during apoptosis, which was caused by high expression levels of anti-apoptotic proteins molecules, Bcl-2 family, and inhibitor of apoptosis proteins (IAP) in HCC cells. Finally, anti-apoptotic pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt signaling, are aberrantly activated in various tumor cells, thus also contributing to TRAIL resistance. Efforts have been made to sensitize TRAIL-resistant HCC cells to the cytotoxicity of TRAIL, such as the combination therapy TRAIL with chemotherapeutic agents. For example, Butein sensitizes HCC cells HepG2 and Hep3B to TRAIL-induced apoptosis via extracellular signal-regulated kinase/Sp1-dependent DR5 up-regulation and NF-κB inactivation. The combination of bortezomib, a proteasome inhibitor, and TRAIL restored the sensitivity of HCC cells including Huh-7, Hep3B, and Sk-Hep-1 to TRAIL-induced apoptosis in part through the inhibition of the PI3K/Akt pathway. Lupeol sensitizes TRAIL therapy-resistant pancreatic cancer cells AspPC-1 for TRAIL therapy by suppressing the expression level of cFLIP and activating caspase-8. Our results also supported the combina-
tion anti-tumor effect of lupeol and TRAIL in the treatment of HCC. However, whether the mechanisms of lupeol sensitizing HCC cells to TRAIL treatment are similar as those in other tumor cells warrants further investigation. Our results also showed that the combination treatment with lupeol and rTRAIL specifically increased the percentage of cells in late stage apoptosis, which suggested the possible role of lupeol in facilitating the progress of early to late stage apoptosis.

Taken together, our results demonstrated that lupeol could selectively and effectively suppress cell growth and induce apoptosis in HCC cells in vitro. It could also suppress HCC tumor growth in vivo in a HCC murine model. Lupeol was also shown to sensitize HCC cells to low-dose rTRAIL treatment. Therefore, our results support the notion that lupeol alone or as an adjuvant to therapeutic agents could be developed as a potential agent for treating HCC.

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