Acacetin (5,7-dihydroxy-4'-methoxyflavone) exhibits *in vitro* and *in vivo* anticancer activity through the suppression of NF-κB/Akt signaling in prostate cancer cells

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Abstract. Acacetin (5,7-dihydroxy-4'-methoxyflavone) is a flavonoid compound with antimutagenic, antiplasmodial, antiperoxidant, anti-inflammatory and anticancer effects. However, the molecular targets and pathways underlying the anticancer effects of acacetin are yet to be elucidated. In this study, we investigated whether acacetin induces apoptosis in the human prostate cancer cell line, DU145. The results of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays revealed that cell viability decreased in a dose- and time-dependent manner in response to acacetin. 4',6-Diamidino-2-phenylindole (DAPI) staining revealed that chromatin condensation significantly increased in a dose-dependent manner. Flow cytometric analysis indicated that acacetin suppressed the viability of DU145 cells by inducing apoptosis. Western blot anlaysis of various markers of signaling pathways revealed that acacetin targets the Akt and nuclear factor (NF)-κB signaling pathways by inhibiting the phosphorylation of IκBα and NF-κB in a dose-dependent manner. Consistent with its ability to induce apoptosis, the acacetin-mediated inhibition of the pro-survival pathway, Akt, and of the NF-κB pathway was accompanied by a marked reduction in the levels of the NF-κB-regulated anti-apoptotic proteins, Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP), as well as of the proliferative protein, cyclooxygenase (COX)-2. We further evaluated the effects of acacetin on prostate cancer using mice subcutaneously injected with DU145 prostate cancer cells. The acacetin-treated nude mice bearing DU145 tumor xenografts exhibited significantly reduced tumor size and weight, due to the effects of acacetin on cancer cell apoptosis, as determined by terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay. Our findings suggest that acacetin exerts antitumor effects by targeting the Akt/NF-κB signaling pathway. Rurther investigations on this flavonoid are warranted to evaluate its potential use in the prevention and therapy of prostate cancer.

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Introduction

Prostate cancer is the leading cause of cancer-related mortality among males in economically developed countries (1); indeed, in the United States, it is only second to lung cancer as the most frequently diagnosed type of cancer in males (2). Prostate cancer is considered malignant as it is a mass of cells that can invade other parts of the body. Currently, no therapies are curative after the cancer invades beyond the gland, metastasizes to the bone and lymph nodes, and becomes androgen-refratory (3). Most of the androgen-dependent stages of prostate cancer respond well to androgen ablation therapy (4). However, during hormonal therapy, androgen-independent tumor cells eventually emerge, leading to clinical relapse (5). No effective therapy is available for such cases, and although hormonal therapy is commonly used alone or in combination with other therapies (6), it is ultimately unsuccessful. A number of cancer types respond to chemotherapy at the initiation of treatment; however, the ability of cancer cells to become resistant to chemotherapeutic drugs remains a significant impediment to successful chemotherapy (7). Therefore, identifying novel anticancer agents and strategies is important.

In general, natural or synthetic chemical agents are employed in cancer chemoprevention to reverse, suppress or prevent cancer progression (8). Flavonoid compounds include a number of chemical subgroups, such as flavonols, procyanidins or anthocyanidins, a broadly distributed class of plant pigments (9). Acacetin (5,7-dihydroxy-4'-methoxyflavone) (Fig. 1) exerts antimutagenic (10), antiplasmodial (11,12), antiperoxidant (13), anti-inflammatory (14) and anticancer effects by suppressing the invasion and migration of human cancer cells (15,16). Acacetin has also been shown to exert an antiproliferative effect by inducing apoptosis and blocking cell cycle progression (8,17). However, the mechanisms underlying the anticancer effects of acacetin in human prostate tumors remain to be elucidated.

The nuclear factor (NF)-κB and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways are major components of the apoptotic machinery, and members of the NF-κB family play an important role in the development and progression of several human malignancies (18). The NF-κB family is composed of five members, RelA, RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (19,20). The activity of NF-κB is primarily regulated by interactions with inhibitory IκBα proteins. Under normal conditions, the typical NF-κB dimers (p50/p65) are bound to inhibitory IκBα proteins, which sequester inactive NF-κB complexes in the cytoplasm. The degradation of IκBα proteins is initiated through phosphorylation by the IκB kinase (IKK) complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit, IKKγ. Phosphorylated IκBα is targeted for ubiquitination and proteasomal degradation, which releases the bound NF-κB dimers. The nuclear localization signals of the NF-κB protein are exposed to allow nuclear translocation and transcriptional activation, ultimately inducing the expression of a number of target genes involved in cell growth, differentiation, inflammatory responses and the regulation of apoptosis (21). NF-κB has been implicated in oncogenesis (22). The constitutive activation of NF-κB has been reported, not only in androgen-independent prostate cancer cell lines, but also in prostate cancer tissues (23,24), suggesting a pivotal role of NF-κB in the progression of prostate cancer.

The PI3K/Akt signaling pathway is a potent survival pathway that mediates resistance to the apoptotic effects of chemotherapeutic drugs and radiation therapy in a variety of cancer types (25). The activation of membrane kinases, such as epidermal growth factor receptor (EGF-R) and insulin-like growth factor receptor (IGF-R) by external growth factors initiates the activation of PI3K and related intracellular pathways (26). Once activated, Akt, a major downstream target of PI3K, transduces signals from growth factors and oncogenes to downstream targets that control essential tumor-associated cellular processes, including cell growth, cell cycle progression, survival, migration, tissue invasion and angiogenesis (27). In addition, Akt directly regulates NF-κB activation through the phosphorylation of p65 by IKK (28,29). Therefore, Akt may also exert some of its pro-survival effects by interacting with other pathways or by exerting effects on nutrient uptake and metabolism.

In this study, we report the *in vitro* and *in vivo* anticancer activity of acacetin in prostate cancer. This study broadens the potential medicinal applications of acacetin, a natural compound that may serve as a novel therapeutic agent for human prostate cancer.

Materials and methods

Chemicals, drugs and antibodies. Acacetin was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C. RPMI-1640 medium, penicillin-streptomycin, trypsin-EDTA and fetal bovine serum (FBS) were purchased from HyClone Laboratories Inc. (Logan, UT, USA). 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were obtained from Sigma-Aldrich. Antibodies against Bax, Bcl-2, β-actin, p53, Akt, phospho-Akt (Ser473), phospho-glycogen synthase kinase (GSK)-3β (Ser9), IκBα, phospho-IκBα (Ser32), phospho-NF-κB p65 (Ser536), X-linked inhibitor of apoptosis protein (XIAP), cyclooxygenase (COX)-2 and goat anti-rabbit horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, MA, USA). Cell lysis buffer and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The DeadEnd™ fluorometric terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was purchased from Promega (Madison, WI, USA).

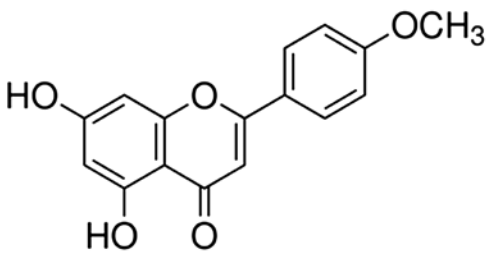

Figure 1. Structure of acacetin (5,7-dihydroxy-4'-methoxyflavone) ($C_{16}H_{12}O_5$, molecular weight, 284.26).

Cell lines and culture. The human prostate carcinoma cell line, DU145, was purchased from the Korean Cell Line Bank (Seoul, Korea), and maintained in RPMI-1640 medium supplemented with 10% FBS and penicillin-EDTA under standard culture conditions, at 37˚C with 95% humidified air and 5% CO_2. The culture medium was renewed every two to three days. For acacetin treatment, DU145 cells were seeded at a density of ~$3x10^4$ cells/cm^2 in a 175-cm^2 flask and allowed to adhere overnight.

Cell viability assay. The anticancer effects of acacetin were assessed by MTT assay. DU145 cells were seeded in a 96-well plate at a density of $2x10^4$ /ml and a volume of 200 μl/well. After 24 h of incubation, the cells were treated with 6.25, 12.5, 25, 50 or 100 μM acacetin for either 24 or 48 h in triplicate. Following treatment, the medium was discarded, followed by the addition of 40 μl of a 5 mg/ml MTT solution and incubation for a further 2 h. The medium was then aspirated and the formazan product generated by viable cells was solubilized with the addition of 100 μl of DMSO. The absorbance of the solutions at 595 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of viable cells was estimated in comparison to the untreated control cells.

Nuclear staining. To quantify acacetin-induced apoptotic cell death, the DU145 cells were treated with either 12.5 or 25 μM acacetin for 24 h. Following treatment, the cells were fixed with 4% paraformaldehyde containing 0.1% Triton X-100 and stained with DAPI for 30 min at room temperature. The cells were washed twice with PBS and examined under a fluorescence microscope (IX71; Olympus Co., Tokyo, Japan).

Western blot analysis. Cells were grown in culture flasks under the same conditions described above and treated with 12.5 or 25 μM acacetin for 24 h. Cells were washed briefly with cold PBS and treated with trypsin-EDTA. Cell pellets were

obtained by centrifugation, lysed in lysis buffer (Invitrogen Life Technologies) and centrifuged at 15,000 rpm for 5 min at 4˚C to obtain whole-cell lysates. Protein concentration was determined using the Bradford protein assay (Bio-Rad), and the samples were stored at -80˚C in small aliquots. Protein extracts (50 μg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred pmto nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated at room temperature for 1 h in a 5% non-fat milk powder solution in Tris-buffered saline (TBS) to block non-specific reactivity. Each membrane was incubated overnight with appropriate primary antibodies at 4˚C and washed with a TBS with Tween-20 (TBS-T) solution. Subsequently, the membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG for 2 h. After washing the membrane three times for 10 min in TBS-T, bands were detected using ECL western blotting detection reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. β-actin was used as a loading control.

Annexin V apoptosis assay. The Annexin V/propidium iodide (PI) assay was performed following the manufacturer's instructions (Becton-Dickinson, San Jose, CA, USA). Briefly, the DU145 cells were treated with or without 12.5 or 25 μM acacetin for 24 h, washed with cold PBS, and incubated with Annexin V and PI in binding buffer at room temperature for 15 min in the dark. Samples were analyzed using a FACSCalibur™ flow cytometer (Becton-Dickinson). All analyses were performed in triplicate.

Animals and in vivo xenograft tumor model. Five-week-old male BALB/c nude mice (nu/nu) were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). Experiments on animals were performed in accordance with the Guidelines for the Care and Use of Animals of the Kongju National University Animals Care Committee (Chungcheongnam-do, Korea). Mice were maintained under a 12-h light/dark cycle, and housed under controlled temperature (23±3˚C) and humidity (40±10%) conditions. Mice were allowed access to laboratory pelleted food and water *ad libitum*.

DU145 cells were injected subcutaneously ($1x10^7$/0.2-ml medium/animal) with a 27-gauge needle into the right flank. When the tumors were palpable, mice were randomly assigned into three groups of five mice in each. Acacetin was orally administered three times per week at a dose of 25 or 50 mg/kg body weight, while the vehicle-treated mice were orally administered distilled water. The weight and tumor size were monitored twice per week. The tumor sizes were measured using vernier calipers and calculated using the following equation: size (mm^3) = 0.5 x length (mm) x $width^2$. Mice were sacrificed 48 days after treatment. The liver and kidneys from each mouse were excised for histopathological examination, and the tumors were also excised to measure tumor wet weight. A portion of the tumor was embedded in paraffin and used for TUNEL assay.

TUNEL assay. Apoptotic cell death was observed using a Promega DeadEnd™ Colorimetric TUNEL system kit according to the manufacturer's instructions. Briefly, tumor tissues were fixed in 10% formalin overnight and embedded in paraffin. These blocks were cut into 5-μm-thick slices. The sections were deparaffinized and hydrated by sequential immersion in xylene and graded alcohol solutions. The tumor sections were visualized using 3'-diaminobenzidine tetrahydrochloride (DAB) solution. The sections were stained with methyl green, treated with a mounting reagent and observed under a microscope (x200).

Histological examination. The excised livers and kidneys were immediately fixed in 10% neutral-buffered formalin and, after embedding in paraffin, cut into 5-μm-thick sections. Following hematoxylin and eosin (H&E) staining, the sections were examined under a light microscope (x200).

Statistical analysis. The results are expressed as the means ± standard deviation (SD). Differences between the mean values for the groups were assessed by a one-way analysis of variance (ANOVA) and Dunnett's t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Induction of DU145 cell death by acacetin. The antiproliferative effects of acacetin on DU145 prostate cancer cells were determined by MTT assay. The cells were treated with 0, 6.25, 12.5, 25, 50 or 100 μM acacetin for 24 or 48 h. As shown in Fig. 2A, acacetin inhibited DU145 cell proliferation in a time-dependent manner. Treatment with 12.5, 25, 50 and 100 μM acacetin for 24 h or 6.25, 12.5, 25, 50 and 100 μM for 48 h resulted in a significant decrease in cell viability compared witht the control group ($P<0.05$). Cell mortality increased by 50% upon treatment with 25 μM acacetin for 48 h. These results suggest that acacetin induces cell death and inhibits cell proliferation.

Induction of DU145 apoptosis by acacetin. DNA damage was observed in DU145 cells, as indicated by morphological changes in the nuclei. The presence of chromatin condensation in the acacetin-treated cells was detected using a fluorescence microscope (x200). DAPI forms fluorescent complexes with double-I banded DNA, and stained nuclei show bright fluorescence under a DAPI filter. The cells were treated with 0, 12.5 or 25 μM acacetin for 24 h. As shown in Fig. 2B, characteristic apoptotic features were observed in the DU145 cells treated with acacetin, including chromatin condensation, convoluted nuclei with cavitations, nuclear fragmentation, and apoptotic bodies. Chromatin condensation and the formation of apoptotic bodies, which are characteristics of apoptosis, were not observed in the untreated cells. These results suggest that acacetin induces the condensation and formation of apoptotic bodies.

Inhibitory effects of acacetin on Akt kinase activation. We examined the effects of acacetin on the Akt cell survival pathway. The DU145 cells were treated with acacetin for 24 h, cell lysates prepared and the phosphorylation of Akt at Ser473 was determined by western blot analysis. The results revealed that the treatment of DU145 cells with acacetin for 24 h decreased the phosphorylation of Akt at Ser473 in a

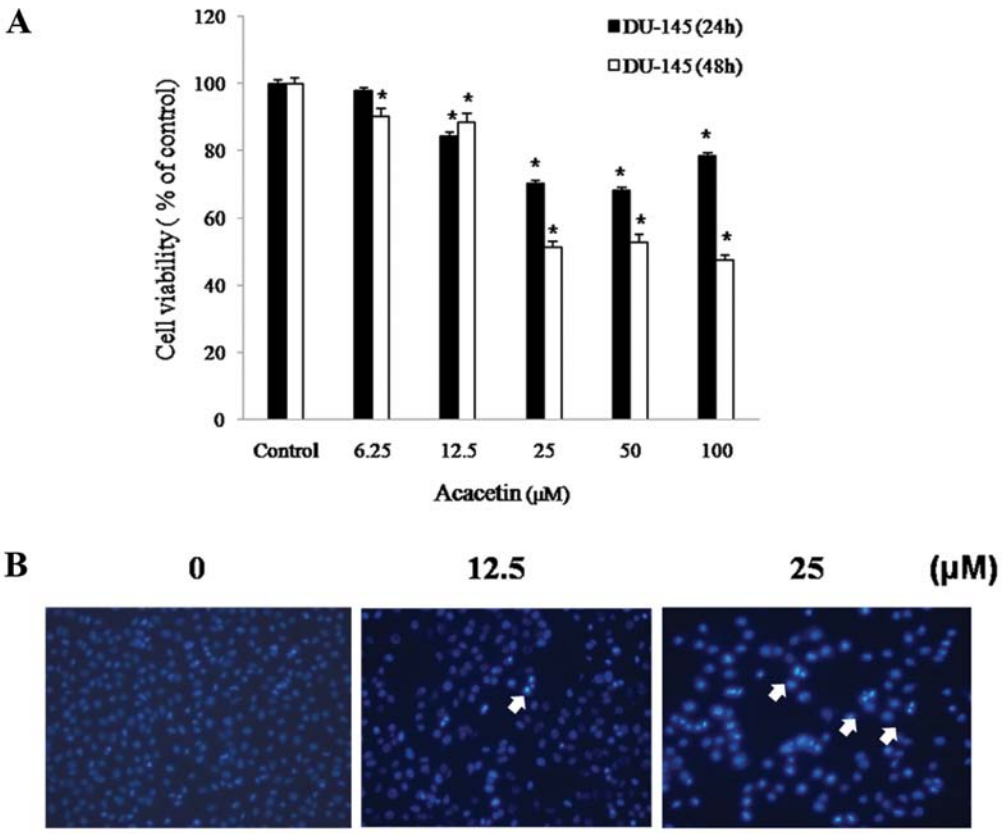

Figure 2. Effects of acacetin on cell viability and apoptosis. (A) DU145 cells were treated with acacetin (0, 6.25, 12.5, 25, 50 and 100 μM) for 24 or 48 h, and cell viability was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results are shown as the means ± standard deviation (SD) of two independent experiments performed in triplicate. Significance was determined by a Dunnett's t-test with $^{*}P<0.05$ considered to indicate a statistically significant difference compared with untreated control cells. (B) DU145 cells were treated with acacetin (0, 12.5 and 25 μM) for 24 h, and apoptotic bodies stained with 4',6-diamidino-2-phenylindole (DAPI) (n=4, means ± SD from triplicate separated experiments). The arrows indicate chromatin condensation in DU145 cells. Cleaved nuclei were examined under a fluorescence microscope (x200).

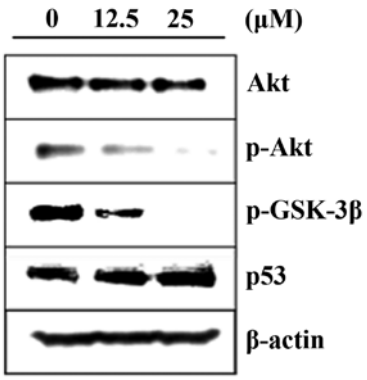

Figure 3. Effect of acacetin on Akt phosphorylation and signaling in DU145 cells. DU145 cells were treated with acacetin (0, 12.5 and 25 μM) for 24 h and cells were harvested to measure protein levels of Akt, p-Akt, p-GSK-3β and p53 by western blotting. We used the housekeeping protein β-actin as a positive loading control in all experiments. Three independent repetitions of the experiment were performed.

concentration-dependent manner, although the total level of Akt remained unchanged (Fig. 3). The expression of downstream effectors of Akt, such as p-GSK-3β and p53, was also evaluated. The total protein concentration of p-GSK-3β was decreased, and that of p53 was increased in the acacetin-treated DU145 cells. These results indicate that acacetin induces apoptosis through the inhibition of Akt activation.

Inhibitory effects of acacetin on NF-κB activation. To confirm that NF-κB is involved in acacetin-induced apoptosis, we examined the expression levels of NF-κB and NF-κB-regulated genes in acacetin-treated cells. As shown in Fig. 4A, our results indicated that acacetin treatment (0, 12.5 and 25 μM) markedly reduced the phosphorylation of IκB, and NF-κB activity in DU145 cells. NF-κB downstream effectors such as, XIAP, COX-2, Bax and Bcl-2 are key mediators of apoptotic death and cell cycle arrest. Therefore, we examined the effects of acacetin on the protein levels of Bax, Bcl-2, COX-2 and XIAP by western blot analysis. Acacetin treatment markedly increased the levels of Bax. In addition, decreased levels of XIAP, COX-2 and Bcl-2 were detected in the acacetin-treated cells (Fig. 4B). These results suggest that the inhibition of NF-κB activation may be the mechanism underlying acacetin-induced apoptosis in DU145 cells.

Induction of apoptosis in DU145 cells by acacetin. To determine the effectd of acacetin treatment on the induction of apoptosis in DU145 cells, we treated the cells with various concentrations of acacetin and assessed the percentage of apoptotic cells by Annexin V/PI double staining (Fig. 5). The cells in the lower right (LR) quadrant of the histogram represent the number

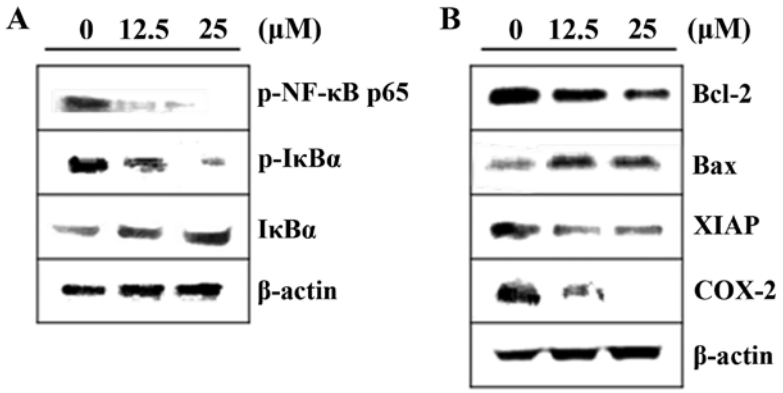

Figure 4. Effect of acacetin on nuclear factor (NF)-κB signaling in DU145 cells. DU145 cells were treated with acacetin (0, 12.5 and 25 μM) for 24 h. (A) Protein levels of total IκBα, p-IκBα and p-NF-κB, as determined by western blot analysis. (B) Protein levels of NF-κB target genes of Bcl-2, Bax, X-linked inhibitor of apoptosis protein (XIAP) and cyclooxygenase (COX)-2, as determined by western blot analysis. The housekeeping protein β-actin served as a positive loading control. Three independent repetitions of the experiment were performed.

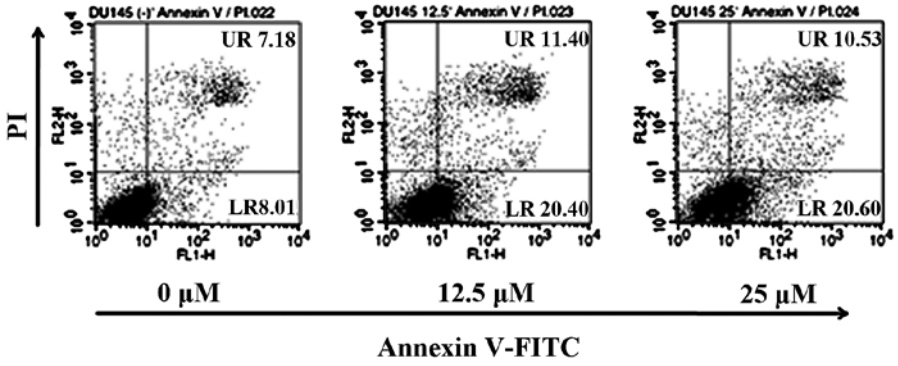

Figure 5. Effect of acacetin on Annexin-positive apoptotic DU145 cells. Cells were treated with acacetin (0, 12.5, 25 μM) in complete medium for 24 h and harvested for analysis by FACS using the Annexin V/propidium iodide (PI) assay, following the manufacturer's instructions. The lower right (LR) quadrant of the FACS histograms indicates the percentage of early apoptotic cells (Annexin V stained cells), and the upper right (UR) quadrant indicates the percentage of late apoptotic cells (Annexin V + PI stained cells). Representative staining profiles for 10,000 cells per experiment are shown.

of early apoptotic cells, while those in the upper right (UR) quadrant of the histogram represent the cells in late apoptosis. Treatment of the DU145 cells with acacetin for 24 h induced a marked, dose-dependent induction of both the early and late stages of apoptosis. Acacetin treatment increased the number of apoptotic cells from 15.19% in the untreated cell group to 31.13% in the group treated with 25 μM acacetin. These data suggest that the induction of apoptosis is a key mechanism underlying the acacetin-induced inhibition of DU145 cell viability (Fig. 2).

Inhibition of tumor growth by acacetin in nude mice. Following the demonstration of the antitumor potential of acacetin in prostate cancer cells *in vitro*, we examined the *in vivo* effects of acacetin on prostate tumor growth using a DU145 prostate cancer xenograft model. Mice were assigned to three groups of five mice in each, and treated with various doses of acacetin (0, 25 or 50 mg/kg). None of these doses of acacetin had any detectable toxic effect, and there were no statistically significant effects on body weight, behavior, or the appearance of the mice (data not shown). As shown in Fig. 6A, tumor size was significantly reduced in the mice treated with 25 or 50 mg/kg acacetin compared with the control mice (P<0.05). On day 29 of acacetin treatment, an important reduction in the tumor size was observed compared with the control. This trend persisted over time and became more pronounced at 40 days of acacetin treatment. On day 48, mice were sacrificed and the tumors excised. Compared with the control, acacetin treatment significantly reduced the mean tumor weight (Fig. 6B). As shown in Table I, the groups treated with acacetin showed significant reductions in tumor size on day 48; 52.00% for the 25 mg/kg and 57.90% for the 50 mg/kg group (both P<0.05 compared with the control group, 0 mg/kg). Moreover, we assayed tumor tissues with TUNEL so as to examine apoptotic cell death. As shown in Fig. 6C, an increase in the number of TUNEL-positive cells was observed in the mice treated with acacetin compared with the control mice (P<0.05). These findings confirmed that the treatment of mice with DU145 tumors with acacetin significantly inhibited tumor growth by inducing the apoptosis of the tumor cells.

Toxicity evaluation of acacetin in liver and kidney tissues. To ensure the safety of acacetin when administered as a chemotherapeutic agent, the mice were sacrificed at the end of the experiment, and liver and kidney tissues were removed and fixed in formalin for histopathological evaluation by H&E

Table I. Inhibitory effects of acacetin on DU145 prostate tumor size.

	Pre-treatment		Post-treatment		
Acacetin dose	n	Size (mm^3)	n	Size (mm^3)	Inhibition rate[b] (%)
0 mg/kg[a]	5	12.79	5	1365.79	
25 mg/kg	5	12.91	5	655.55	52.00
50 mg/kg	5	13.86	5	575.03	57.90

[a]Control group; [b]Data are expressed as a percentage relative to the control.

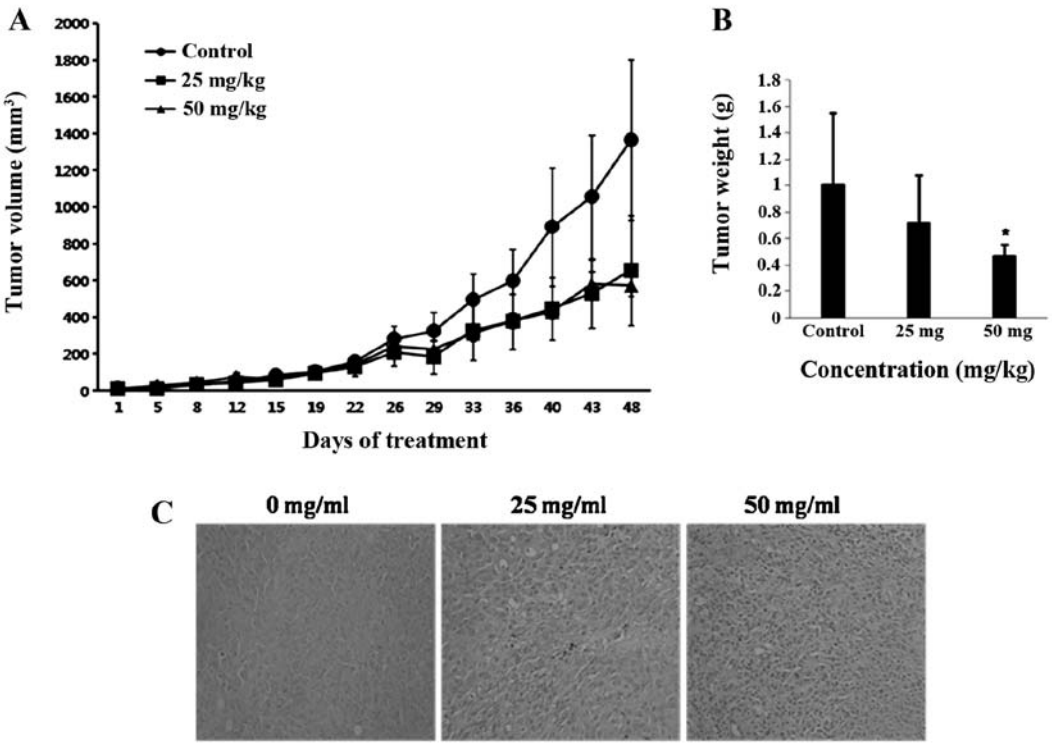

Figure 6. Acacetin inhibits DU145 prostate tumor growth and induces apoptosis. Male BALB/c nude mice received an injection of DU145 cells and were divided into three groups. Acacetin was administered at a dose of 25 or 50 mg/kg three times per week, for a total 21 injections. On day 48, mice were sacrificed and tumors excised. (A) Acacetin significantly reduced tumor size starting from the 22nd day of treatment. (B) The mean tumor weight in the acacetin group was reduced compared with the control group. (C) Nude mice were treated with acacetin (0, 25 or 50 mg/kg) for 48 days and tumors analyzed by terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay. Paraffin-embedded tumors were sectioned to a thickness of 5 μm. The slides were examined under a microscope and photographed (x200).

staining (Fig. 7). No pathological change was observed in the acacetin-treated group compared with the control group.

Discussion

Compelling evidence suggests that the tumorigenic growth of prostate cancer depends on the disruption of the normal apoptotic process (30). Both the incidence of prostate cancer and the associated mortality rate are increasing steadily. Currently available chemotherapeutic agents for the treatment of prostate cancer are associated with various side-effects and the development of resistance. Therefore, non-toxic and more selective pharmacotherapies that target prostate cancer are required.

Over the past decade, a number of naturally occurring dietary agents of reduced toxicity have been reported to induce apoptosis and inhibit tumor growth. One of these, acacetin, exhibits a number of biological effects, including anticancer activity (8,15,16,17,31); however, its underlying mechanisms of action remain unknown. In this study, we demonstrate that acacetin induces the apoptosis of DU145 human prostate cancer cells by inhibiting the activation of the Akt-NF-κB signaling pathway. We found that acacetin suppressed constitutive NF-κB activation through the inhibition of Akt in DU145 human prostate carcinoma cells. In addition, acacetin altered the expression of signaling effectors, including GSK-3β, IκB, Bcl-2 and Bax, as well as that of inhibitors of apoptosis (IAP) family members, such as XIAP and COX-2 (Figs. 3 and 4). Furthermore, we provide evidence that acacetin can effectively inhibit the growth of prostate cancer tumors without overt toxicity. These results provide mechanistic insight into

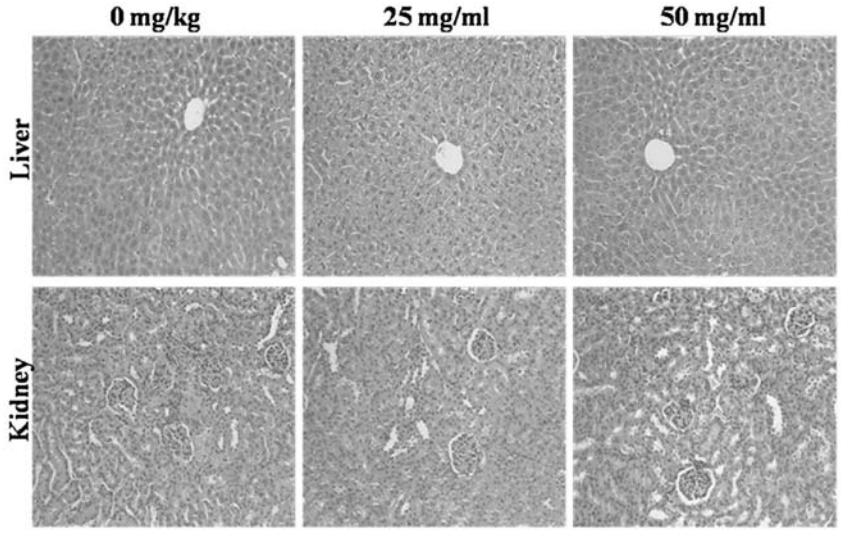

Figure 7. Histological observation of acacetin-treated nude mice. Male BALB/c nude mice received an injection of DU145 cells and were divided into three groups. Acacetin was administered at a dose of 25 or 50 mg/kg three times per week, for a total 21 injections. On day 48, mice were sacrificed, and tumors excised and evaluated by hematoxylin & eosin (H&E) staining. The dose of acacetin had no detectable toxicological effect on nude mice.

the *in vivo* and *in vitro* anticancer effects of acacetin, which we suggest are mediated, at least in part, by blocking the proliferative and anti-apoptotic effects of NF-κB signaling through the reduction of the translocation of this protein complex to the nucleus and inhibition of Akt phosphorylation.

In order to evaluate the cytotoxicity of acacetin, an MTT assay was performed to determine cell viability. Acacetin induced a potent time- and a dose-dependent decrease in DU145 cell number. Overall, the data suggest that acacetin inhibits the growth of human prostate cancer cells. This result confirms an earlier report on the antiproliferative effects of acacetin in the AGS cancer cell line (31).

Apoptosis, otherwise known as programmed cell death, is characterized by a number of well-defined features, such as condensation and fragmentation of chromatin, inter-nucleosomal DNA cleavage, caspase activation and the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (32). The induction of apoptosis is one of the most effective approaches in cancer therapeutics. To determine whether the acacetin-induced cell death in DU145 cells involved apoptosis, we performed DAPI staining and flow cytometric analysis. As shown in Figs. 2 and 5, acacetin was a potent inhibitor of cell viability and induced the rapid induction of apoptosis, concurrent with chromatin condensation and the apoptotic appearance of DU145 cells, in agreement with previous reports on human prostate cancer cells (8,16). Further studies are required to elucidate the mechanisms behind the reduced cell viability and the induction of apoptosis in DU145 cells by acacetin.

The Akt signaling pathway is a critical component in the regulation of cell growth, survival and apoptosis (33). The aberrant activation of the Akt pathway and, hence, of these tumor-associated pathways, has been reported in a number of human malignancies (34). Furthermore, Akt signaling activates the NF-κB signaling pathway to promote the resistance of cancer cells to apoptosis (35). Therefore, the specific inhibition of the Akt pathway may be an effective approach to prevent and treat malignancies. In the present study, we demonstrated that Akt phosphorylation was inhibited and the expression of p53 was increased by acacetin treatment. Moreover, the acacetin-mediated suppression of Akt phosphorylation in DU145 cells was coupled to the inhibition of GSK-3β. These data suggest that the inhibition of the activation of Akt is potentially one of the underlying mechanisms of acacetin-induced apoptosis in DU145 cells.

NF-κB is a family of dimeric transcription factors that regulate diverse biological processes, including immune responses and cell growth and survival (36,37). In response to most activating stimuli, NF-κB signaling occurs through the sequential activation of IKK, the phosphorylation of IκBα at Ser32 and Ser36, leading to IκBα degradation, and the translocation of NF-κB to the nucleus, where it regulates the transcription of a series of genes, including those that promote cell proliferation and survival (38). In this study, we found that acacetin was a potent inhibitor of NF-κB activation in DU145 cells. Acacetin inhibited NF-κB phosphorylation and IκB phosphorylation and degradation, eventually leading to the inhibition of NF-κB nuclear translocation. Acacetin also affected the levels of NF-κB-regulated proteins involved in apoptosis (Bax), anti-apoptosis (Bcl-2 and XIAP) and proliferation (COX-2), thereby suppressing cell proliferation and inducing apoptosis in DU145 cells.

The *in vivo* anticancer efficacy of acacetin was substantiated by our experiments on DU145 tumor-bearing nude mice. Acacetin was administered three times per week at a dose of 25 or 50 mg/kg. As shown in Table I, the mice administered with acacetin showed a marked reduction in tumor size at day 48: 52.00% for the 25 mg/kg group and 57.90% for the 50 mg/kg group compared with the control group. This *in vivo* antitumor effect correlated with the increased levels of apoptosis in the tumor cells of the acacetin-treated group, as supported by the increased DNA fragmentation observed in the TUNEL-positive cells. The *in vivo* results support our *in vitro* studies and suggest that acacetin induces apoptotic cell death in DU145 prostate tumor cells. Evaluation of the potential toxic effects of acacetin on healthy tissues is an important

factor considered during the development of novel anticancer drugs, aiming to prevent side-effects of the tested drug on non-targeted cells, such as severe DNA damage (39). As shown in Fig. 7, none of the mice in the three groups showed any significant clinical symptoms during the study period. Therefore, acacetin showed no significant toxicity in mice at the doses tested (<50 mg/kg).

In conclusion, we provide evidence that acacetin significantly attenuates tumor progress, and that the antitumor effects of this flavonoid are mediated by inhibiting the phosphorylation of Akt and reducing NF-κB DNA binding. The present study provides a molecular basis for the use of acacetin as a cancer chemopreventive and chemotherapeutic agent. Overall, our results indicate that flavonoid compounds, such as acacetin, may be useful in the treatment of prostate cancer.

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