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Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways

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ABSTRACT

The progression of prostate cancer is associated with escape from cell cycle arrest and apoptosis under androgen-depleted conditions. Here, we found that geraniol, a naturally occurring monoterpene, induces cell cycle arrest and apoptosis in cultured cells and tumor grafted mice using PC-3 prostate cancer cells. Geraniol modulated the expression of various cell cycle regulators and Bcl-2 family proteins in PC-3 cells *in vitro* and *in vivo*. Furthermore, we showed that the combination of sub-optimal doses of geraniol and docetaxel noticeably suppresses prostate cancer growth in cultured cells and tumor xenograft mice. Therefore, our findings provide insight into unraveling the mechanisms underlying escape from cell cycle arrest and apoptosis and developing therapeutic strategies against prostate cancer.

1. Introduction

Prostate cancer is the second leading cause of cancer mortality among men [1,2]. Androgen-ablative therapy is initially effective in the treatment of advanced or metastatic prostate cancer [3–5], which indicates that the growth of prostate cancer depends on androgen. Indeed, accumulating evidence shows that androgen ablation induces cell cycle arrest and apoptosis [6,7]. However, the prostate cancer eventually evolves to acquire novel traits, including the capability to promote androgen-independent growth and a resistance to other therapeutic options, including chemotherapy [8–10]. These observations indicate that prostate cancer cells are reprogrammed to escape from cell cycle arrest and apoptosis under androgen-depleted conditions. Thus, targeting the escape programs of the tumor cells can be a promising strategy for treatment of androgen-independent prostate cancer (AIPC).

Cell cycle is controlled by the periodic regulation of a number of cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitory proteins [11,12]. Aberrant activity of these regulators confers a limitless replicative potential, which is a hallmark of cancer [13,14]. In prostate cancer, deregulations or mutations in the cell cycle regulators are frequently observed and allow the tumor cells to grow under androgen-depleted conditions and eventually stimulate the progression into AIPC [10,15,16]. Thus, these observations indicate that controlling cell cycle can be an attractive strategy for development of prostate cancer therapeutics [17,18]. In fact, several small molecule CDK inhibitors are currently undergoing clinical trials for the treatment of patients with prostate cancer [19–21].

In addition to an uncontrolled cell cycle, evasion of apoptosis is also a hallmark of cancer [13,14]. Small molecule CDK inhibitors have been reported to induce apoptosis in several types of cancer cells [22,23]. Although it is unclear whether cell cycle inhibition is causally involved in apoptosis, these observations suggest that cotargeting cell cycle and apoptosis pathways can be an effective strategy for developing anticancer therapeutics. The deregulation of cell cycle regulators (e.g., cyclins and CDKs) and apoptosis regulators (e.g., Bcl-2 family proteins) is frequently observed in prostate cancer and can contribute to the progression into AIPC and the development of resistance to therapeutic regimens [10,24]. Indeed,

Abbreviations: AIPC, androgen-independent prostate cancer; CDK, cyclin-dependent kinase.

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small molecule CDK inhibitors have an effect of lowering the therapeutic threshold for conventional anticancer therapies [22,25], which suggests that cotargeting cell cycle and apoptosis pathways enhances chemosensitivity.

Geraniol is a natural acyclic monoterpene derived from herb oils [26]. It is considered as generally-recognized-as-safe (GRAS) by FDA [27]. Few papers showed that geraniol possesses chemotherapeutic potentials [28,29]. Therefore, geraniol may be a useful chemical moiety to serve as a lead molecule for anticancer drug development. In addition, an effort to understand the mechanisms underlying the actions of geraniol can provide insight into novel therapeutic target discovery. However, the mechanisms by which geraniol exerts anticancer activity are poorly understood. In this study, we investigated the anticancer effect of geraniol on prostate cancer using the PC-3 cell line, a cell model for AIPC.

2. Materials and methods

2.1. Cell viability assay

MTT and LDH release assays were used to assess the viability of PC-3 cells according to the manufacturers' instructions (Sigma or Promega, respectively). Geraniol was dissolved in ethanol as a vehicle at 0.1% working concentration. The assays were quantitated by measuring the absorbance at 570 (MTT assay) or 490 nm (LDH assay) on a microplate spectrophotometer (ASYS).

2.2. Caspase-3 activity assay

Capase-3 activity was determined using a commercial kit according to the manufacturer's instruction (BioMol). The crude extracts prepared from PC-3 cells were incubated with chromogenic caspase-3 substrates. The absorbance was measured at 405 nm on microplate spectrophotometer.

2.3. Flow cytometry analysis

To analyze cell cycle profiles, PC-3 cells fixed with 70% ethanol were labeled with propidium iodide (50 μg/ml) solution containing RNase A (100 μg/ml). To determine apoptotic cells in the tumor tissues, PE-conjugated antibody to active capspase-3 (BD Biosciences) was used as previously described [30]. To measure cells in proliferation, FITC-conjugated antibody to Ki-67 was used according to the manufacturer's instruction (Thermo Scientific). The cell population isolated from the tumor tissues was used to analyze the ratio of apoptotic and proliferative cells.

2.4. Assessment of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was evaluated using the JC-1 fluorescence probe according to the manufacturer's instruction (Molecular Probes). PC-3 cells were labeled with JC-1 at 2 μM for 30 min at 37 °C and then analyzed by flow cytometry using 488 nm excitation with 530/30 or 585/42 nm bypass emission filters. The cells without red fluorescence were considered as the cells manifesting mitochondrial membrane depolarization.

2.5. Electrophysiology and intracellular Ca^{2+} measurement

Patch clamp experiments were performed in the whole cell configuration using an Axopatch I-D amplifier. Electrode resistance was 2–5 MΩ and 60% of the series resistance was compensated. Internal and extracellular solutions were prepared as previously described [31]. The TRPM8-specfic currents were stimulated by addition of (−)-menthol or geraniol to the bath solution. Current–voltage relationship data were obtained from linear 400-ms voltage ramps from −100 to +100 mV at a holding potential of −60 mV. Intracellular calcium concentration ($[Ca^{2+}]_{cyt}$) was measured as previously described [32].

2.6. Western blot analysis

The total proteins were prepared by incubation with RIPA buffer with protease and phosphatase inhibitor cocktails (Calbiochem). The proteins were resolved in 10%, 12% or 15% SDS–PAGE gels and analyzed with the denoted antibodies. Antibodies to Cyclin D, Cyclin E, Bcl-xL, CDK1, BNIP3 and Bim were obtained from Cell Signaling. Antibodies to Cyclin A, Cyclin B1, p21, p27, Bcl-2, Bcl-w, Bax, CDK 2, CDK 4, and CDK6 were purchased from Santa Cruz.

2.7. Xenograft experiment

Balb/C nude mice were subcutaneously inoculated with 1×10^6 PC-3 cells per mouse in the left inguinal region. Geraniol at concentrations of 0, 12, 60, or 300 mg/kg was daily treated by intratumoral injection. To determine the anti-tumor effect of the combination of geraniol and docetaxel, 20 mg/kg geraniol with or without 2 mg/kg docetaxel was daily treated by intratumoral injection. The tumor size was assessed twice per week using caliper measurements and the measured values were used to calculate the tumor volume for each individual mouse as previously described [33]. Thirty-eight days after challenge of PC-3 cells, the mice were sacrificed to measure tumor weights.

2.8. Statistical analysis

All data were expressed as mean ± SEM. Comparison of means among experimental groups was carried out with ANOVA followed by a post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Geraniol induces cell cycle arrest and apoptosis in PC-3 cells in vitro

Because the anticancer effect of geraniol on prostate cancer has not been examined, we first examined whether geraniol suppresses PC-3 cell growth. MTT assays showed that cell population gradually decreased in relation to concentrations of geraniol (Fig. 1A). The anticancer activity of geraniol was confirmed by LDH release assays. Increased LDH activity was observed in the cells treated with geraniol in a dose-dependent manner (Fig. 1B). To determine the cellular processes underlying geraniol-induced growth inhibition of PC-3 cells, we first analyzed the cell cycle profile of PC-3 cells following treatment with geraniol. The percentage of G1 and/or sub-G1 phase cells was markedly increased in the cells treated with geraniol (Fig. 1C and D). We then investigated whether geraniol triggers apoptosis. Caspase-3 activity was increased in relation to concentrations of geraniol in PC-3 cells (Fig. 1E). We also observed that geraniol noticeably evoked mitochondrial membrane depolarization, an early event for intrinsic apoptosis signaling (Fig. 1F). These findings indicate that geraniol has the ability to induce G1 phase arrest and/or apoptosis.

To ascertain these results, we performed Western blot analysis (Fig. S1A and B). The expressions of four cyclin isotypes (cyclin A, B, D, and E), two of CDK family (CDK1 and CDK4), and two anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-w) are reduced in the cells treated with geraniol. Contrastingly, the expressions of two CDK inhibitory proteins (p21 and p27) and two pro-apoptotic

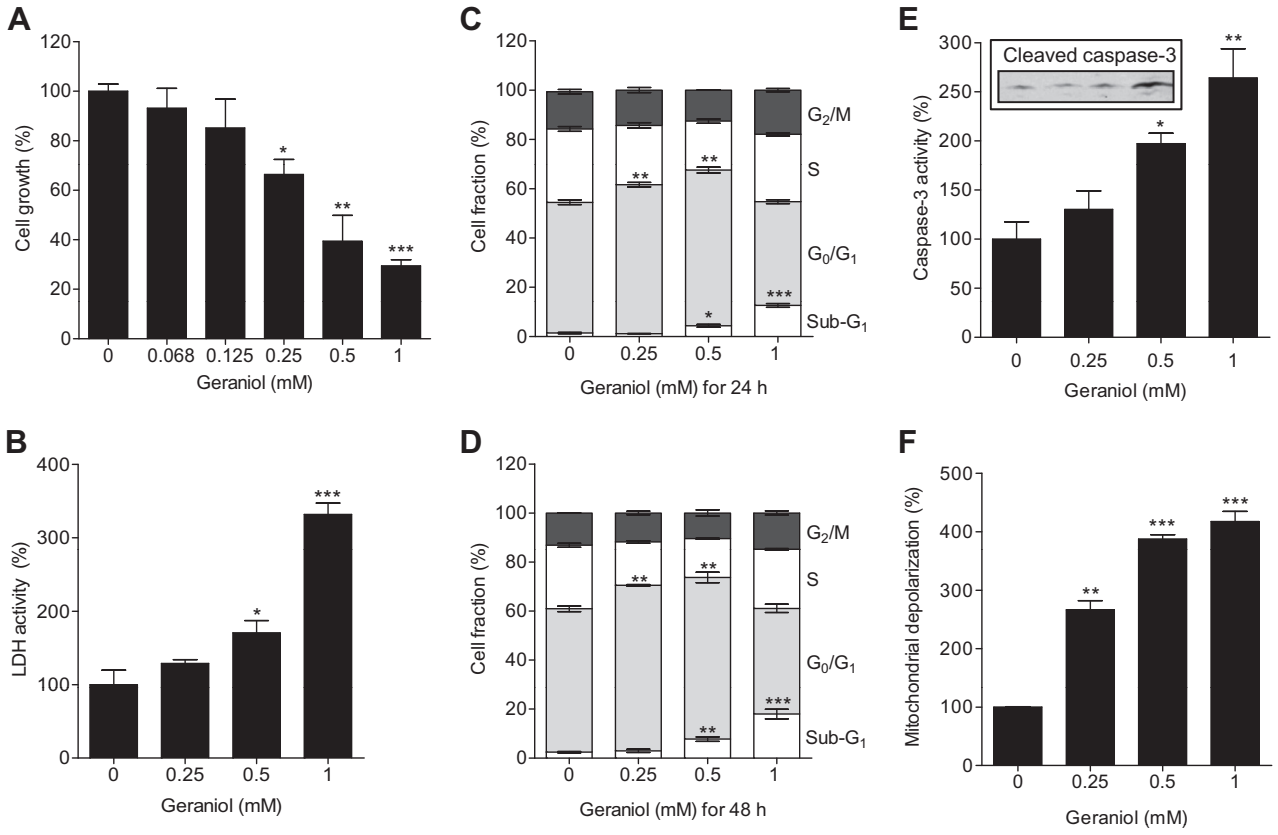

Fig. 1. Geraniol induces cell cycle arrest and apoptosis in PC-3 cells *in vitro*. (A) MTT assays with the cells treated with geraniol for 72 h. Cell growth is expressed as a relative value to that of the untreated cells which is set to 100%. (B) LDH release assays with the cells treated with geraniol for 72 h. LDH activity is expressed as a relative value to that of the untreated cells which is set to 100%. The figures show mean ± SEM ($n = 4$). (C–D) Cell cycle analysis with the cells treated with geraniol for 24 h (C) or 48 h (D). Cell fraction is expressed as the percentage of cells in each phase of the cell cycle. The figures show mean ± SEM ($n = 4$). (E) Caspase-3 assays following treatment with geraniol for 48 h. Caspase-3 activity from untreated cells is expressed as 100%. The figures show mean ± SEM ($n = 6$). Cleaved casapase-3 was assessed by western blot analysis (inset). Loading amount was assessed using anti-GAPDH antibody (data not shown). (F) The measurement of mitochondria membrane potential following treatment with geraniol for 48 h. Mitochondria membrane depolarization is expressed as a relative value to that of untreated cells which is set to 100%. The figures show mean ± SEM ($n = 6$).

Bcl-2 family members (Bax and BNIP3) were markedly elevated under the same conditions. Therefore, our data demonstrate that geraniol inhibits the molecules associated with cell cycle progression and anti-apoptosis and concomitantly activates those favoring cell cycle pause and apoptosis.

3.2. Geraniol inhibits PC-3 cell growth in a xenograft model

To further assess the anticancer activity of geraniol, we performed PC-3 cell xenograft experiments using nude mice. The reductions in tumor volume and weight were obvious in the mice treated with geraniol at 60 or 300 mg/kg (Fig. 2A and B), indicating that geraniol effectively suppresses tumor growth *in vivo*. To determine whether geraniol induces apoptosis and/or inhibits proliferation of tumor cells *in vivo*, we analyzed the percentage of active caspase-3-positive cells and the level of Ki-67, a proliferation marker, using flow cytometry with cells isolated from the tumor tissues. The percentage of apoptotic cells was elevated in the tumor cells treated with 60 or 300 mg/kg of geraniol (Fig. 2C). On the other hand, the expression level of Ki-67 was reduced in the tumor cells treated with 60 or 300 mg/kg of geraniol (Fig. 2D). These data indicate that geraniol induces apoptosis and cell cycle arrest *in vivo*. These results were corroborated by Western blot analysis. The expressions of four cyclin isotypes (cyclin A, B, D, and E), two of the CDK family (CDK1 and CDK4), and Bcl-2 were reduced in the tumor tissues treated with geraniol (Fig. 2E). In contrast, the expression of p21, p27, and Bax were markedly elevated in the tumor tissues.

3.3. Geraniol inhibition of cell growth is independent of TRPM8 calcium channel

Geraniol is structurally related to menthol, an agonist of the TRPM8 calcium channel (Fig. 3A). Indeed, geraniol is known to evoke an increase in $[Ca^{2+}]_{cyt}$ [34]. However, it is unclear whether geraniol activates TRPM8 leading to the $[Ca^{2+}]_{cyt}$ increase. To prove whether geraniol acts as a TRPM8 agonist, we performed the whole-cell patch clamp analysis using HEK-293 cells overexpressing TRPM8. An outwardly rectifying current–voltage relationship, a hallmark of TRPM8, was observed in the cells exposed to 1 mM menthol (Fig. 3B). By contrast, no TRPM8-specific currents were observed by 1 mM geraniol in the cells overexpressing TRPM8 (Fig. 3C). These results demonstrate that geraniol does not act as a TRPM8 agonist.

To provide a clue that geraniol has the ability to induce TRPM8-independent $[Ca^{2+}]_{cyt}$ increase, we measured the effect of geraniol on $[Ca^{2+}]_{cyt}$ in HEK 293 cells, in which TRPM8 is not endogenously expressed [31,35]. Geraniol at 1 mM evoked a robust increase in $[Ca^{2+}]_{cyt}$ in HEK-293 cells (Fig. S2A), indicating that geraniol has the ability to increase $[Ca^{2+}]_{cyt}$ in a TRPM8-independent manner. Additionally, in PC-3 cells, we found that geraniol evokes a very slight increase in $[Ca^{2+}]_{cyt}$ (Fig. S2B). These results indicate that the anticancer activity of geraniol is unrelated to TRPM8 activation.

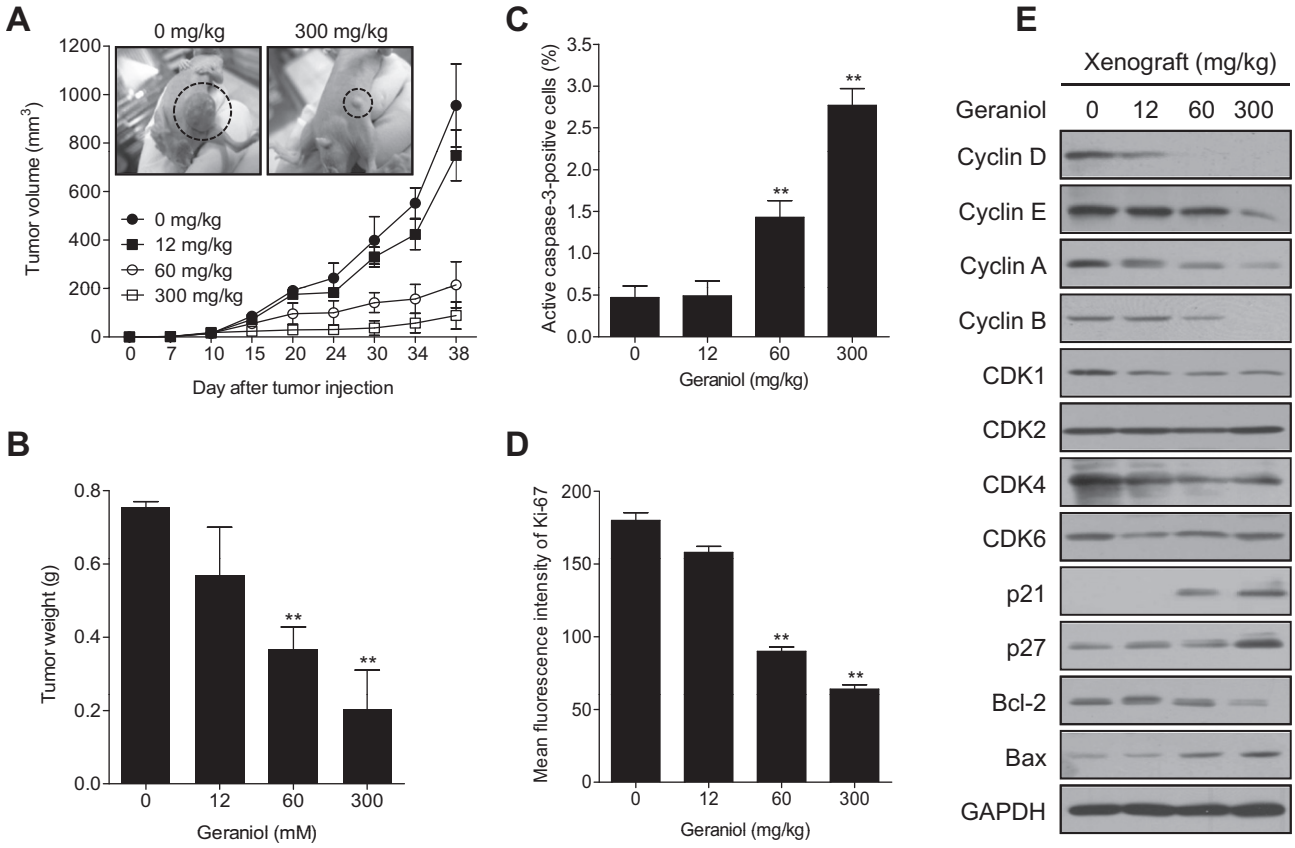

Fig. 2. Geraniol inhibits the growth of PC-3 cells *in vivo*. BALB/c nude mice were subcutaneously inoculated with 1×10^6 PC-3 cells per mouse. When tumor volumes reached approximately 15 mm^3, the mice were treated with geraniol at the indicated concentrations. (A) The tumor volumes were recorded twice per week for 38 day. The inset shows the representative photo images. The figures show mean ± SEM (5 mice per group). (B) At 38 days after xenograft implantation, the mice were sacrificed to determine tumor weight. The figures show mean ± SEM (5 mice per group). (C) Apoptotic cell assays using PE-conjugated antibody to active caspase-3. The bar graph depicts the percentage of apoptotic cells in the tumor tissues. The figures show mean ± SEM ($n = 4$). (D) Proliferative cell assays using FITC-conjugated antibody to Ki-67. The bar graph represents the mean values of Ki-67 fluorescence intensity in the tumor cells isolated from the tumor tissues. The figures show mean ± SEM ($n = 4$). (E) Western blot analysis using the indicated antibodies. The crude extracts were prepared from the xenografted PC-3 cells at 38 days after implantation. Western blots are representative of 3–4 independent experiments.

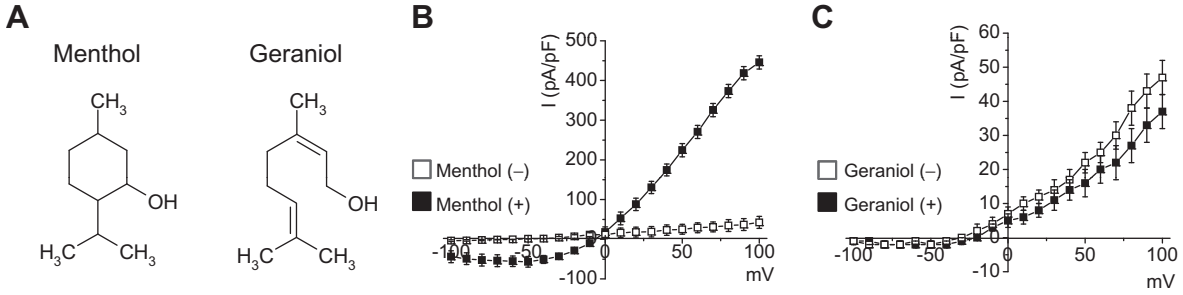

Fig. 3. The anti-tumor activity of geraniol is unrelated to TRPM8 activation. (A) Chemical structures of menthol and geraniol. (B–C) Current–voltage relation (I–V curve) obtained from whole-cell patch clamp analysis. HEK-293 cells overexpressing human TRPM8 were used to determine the electrical responses evoked by 1 mM of menthol (B) or geraniol (C). The figures show mean ± SEM ($n = 12$). Note the different scales of *y*-axis.

3.4. Geraniol increases the chemosensitivity of PC-3 cells in a xenograft model

Small molecule CDK inhibitors, which induce cell cycle arrest and apoptosis, are known to have chemosensitizing activity [22,25]. We first investigated whether geraniol is able to enhance the sensitivity of PC-3 cells to chemotherapeutic agents *in vitro*. Compared to each agent alone, the combination of a chemotherapeutic agent plus geraniol markedly suppressed cell growth (Fig. S3A–F). Because docetaxel is widely used as a chemotherapeutic agent for AIPC treatment in the clinic, we assessed whether geraniol enhances the sensitivity of PC-3 cells to docetaxel *in vivo*. Nude mice were inoculated with PC-3 cells and treated with 20 mg/kg geraniol and/or 2 mg/kg docetaxel. Tumor volumes and weights were noticeably reduced in the group treated with 20 mg/kg of geraniol combined with 2 mg/kg of docetaxel, compared to those groups treated with either agent alone (Fig. 4A and B). We further analyzed the percentage of active caspase-3-positive cells in the tumor tissues. As shown in Fig. 4C, the percentage of apoptotic cells was remarkably elevated in the

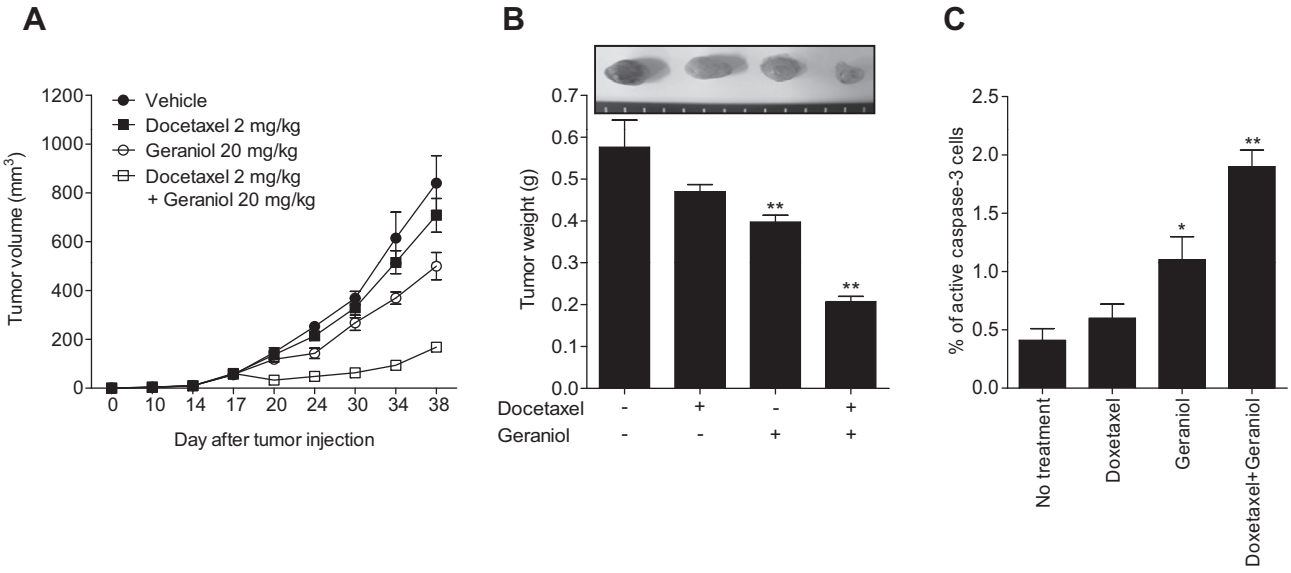

Fig. 4. Geraniol increases the chemosensitivity of PC-3 cells *in vivo*. BALB/c nude mice were subcutaneously inoculated with 1×10^6 PC-3 cells per mouse. When tumor volumes reached approximately 15 mm^3, the mice were treated with 20 mg/kg geraniol and with or without 2 mg/kg docetaxel. (A) Tumor volumes were recorded twice per week for 38 day. The figures show mean ± SEM (5 mice per group). (B) The bar graph represents tumor weight at 38 days after tumor challenge. The figures show mean ± SEM (5 mice per group). The inset is the representative photo image of tumors (C) Apoptotic cell analysis. The bar graph depicts the percentage of apoptotic PC-3 cells, which is determined using PE-conjugated antibody to active caspase-3. The figures show mean ± SEM ($n = 5$).

tumor tissues treated with the 20 mg/kg of geraniol combined with 2 mg/kg of docetaxel, compared to the tumor tissues treated with either agent alone. Therefore, our data suggest that geraniol lowers the therapeutic threshold for conventional anticancer drugs *in vivo*.

4. Discussion

In this study, we demonstrated that geraniol suppresses PC-3 cell growth in culture and xenograft models. Molecular and cellular analyses showed that the anticancer activity of geraniol is attributable to cell cycle arrest and/or apoptosis. In addition, we found that geraniol increases the sensitivity of PC-3 cells to chemotherapeutic agents. Our results indicate that geraniol is a useful chemical moiety for polypharmacological approaches for future development of anticancer drugs [36]. In addition, our findings suggest that geraniol would be a useful chemical tool for approaches to identify novel therapeutic targets for AIPC.

Androgen exerts its actions through the androgen receptor, a crucial effector molecule for development and progression of prostate cancer [37]. Androgen ablation initially suppresses tumor growth via triggering cell cycle arrest and apoptosis in prostate cancer cells [6,7]. However, it acts as a selection pressure to drive cancer cells to evolve evading mechanisms that restore androgen receptor activity [38]. Therefore, prostate cancer cells escape from cell cycle arrest and apoptosis occurring under androgen-ablated conditions. Indeed, prostate cancer cells accumulate the mutations that lead to defects in cell cycle checkpoints and eventually to cell cycle misregulation [10,15,16]. Moreover, anti-apoptotic proteins are up-regulated in high grade or relapsed prostate cancer [24]. Together with these observations, our data highlight the importance of a strategy for cotargeting cell cycle and apoptosis pathways to overcome the bypass mechanisms of AIPC acquired under androgen-depleted conditions.

AIPC is notoriously resistant to available chemotherapeutic regimens and its prognosis is poor [9]. Previously, flavopiridol (a small molecule CDK inhibitor) and docetaxel have shown anti-tumor effects against several types of cancers in clinical settings [39]. Flavopiridol induces cell cycle arrest and apoptosis in cancer cells, suggesting that the therapeutic strategy of cotargeting cell cycle and apoptosis can be useful to increase chemosensitivity [40]. Our study showed that geraniol can be a promising chemosensitizer through cotargeting both cell cycle and apoptosis pathways. Tumor graft experiments demonstrated that geraniol at a sub-toxic dose of geraniol increases the sensitivity of PC-3 cells to docetaxel at a sub-toxic dose. These results indicate that geraniol may provide the insight into circumventing the clinical problem of chemoresistance in treating AIPC. In addition, our findings indicate that icilin would be a valuable chemical probe for future investigation aiming at illuminating the molecular mechanisms underlying chemoresistance of AIPC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.124.

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