

# Activation of AMPK by Pterostilbene Suppresses Lipogenesis and Cell-Cycle Progression in p53 Positive and Negative Human Prostate Cancer Cells

Victor Chia-Hsiang Lin,<sup>†,§</sup> Ya-Chu Tsai,<sup>#</sup> Jia-Ni Lin,<sup>⊥</sup> Ling-Ling Fan,<sup>#</sup> Min-Hsiung Pan,<sup>⊗</sup> Chi-Tang Ho,<sup>△</sup> Jiumn-Yih Wu,<sup>\*,§</sup> and Tzong-Der Way<sup>\*,#,○</sup>

<sup>†</sup>Department of Urology, E-Da Hospital, Kaohsiung, Taiwan

<sup>§</sup>Department of Chemical Engineering, Institute of Biotechnology and Chemical Engineering, I-Shou University, Kaohsiung, Taiwan

<sup>#</sup>Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichung, Taiwan

<sup>⊥</sup>The Ph.D. Program for Cancer Biology and Drug Discovery, College of Pharmacy, China Medical University, Taichung, Taiwan,

<sup>⊗</sup>Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan

<sup>△</sup>Department of Food Science, Rutgers University, New Brunswick, New Jersey, United States

<sup>○</sup>Institute of Biochemistry, College of Life Science, National Chung Hsing University, Taichung, Taiwan

**ABSTRACT:** Prostate cancer is one of the leading causes of cancer death in men in Western countries. Epidemiological studies have linked the consumption of fruits and vegetables to a reduced risk of prostate cancer, and small fruits are particularly rich sources of many active phytochemical stilbenes, such as pterostilbene. As a constituent of small fruits such as grapes, berries, and their products, pterostilbene is under intense investigation as a cancer chemopreventive agent. Using the p53 wild type LNCaP and p53 null PC3 cells, we found that treatment with pterostilbene resulted in dose-dependent inhibition of cellular proliferation, which suggested that the interaction of pterostilbene with the p53 might not fully explain its inhibitory effect on proliferation. In this study, we found that pterostilbene activated AMPK in both p53 positive and negative human prostate cancer cells. Pterostilbene-activated AMPK decreased the activity and/or expression of lipogenic enzymes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). Interestingly, the resolution between apoptosis and growth arrest following AMPK activation is greatly influenced by p53 status. In p53 positive LNCaP cells, pterostilbene blocked the progression of cell cycle at G1 phase by inducing p53 expression and further up-regulating p21 expression. However, pterostilbene induced apoptosis in p53 negative PC3 cells. Our results suggest that pterostilbene may be a functional chemopreventive agent and that dietary exposure to pterostilbene would be helpful for antiprostata cancer activity.

**KEYWORDS:** pterostilbene, AMPK, FASN, p53, prostate cancer cells

## ■ INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in men and the second leading cause of cancer death in the United States.<sup>1</sup> The incidence and mortality of prostate cancer show noticeable geographic variation, being highest in North America and western Europe and lowest in Asia.<sup>2</sup> Although these differences undoubtedly have a genetic component, numerous epidemiological studies support an association between dietary fat intake and prostate cancer risk.<sup>3</sup> Therefore, there is growing interest in targeting metabolic pathways that may be altered during prostate tumorigenesis and prostate cancer progression.

Experts allude to diets rich in fruits and vegetables as a key for keeping the body healthy. Certain groups of compounds, such as polyphenols, have gained popularity as health beneficial constituents in edible plants. Over the past decade, there have been increasing applications for naturally derived phytochemicals as anti-inflammatory and cancer chemopreventive agents. Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a dimethyl ether analogue of resveratrol, is a natural antioxidant predominantly found in blueberries, grapes, and a tree wood, *Pterocarpus marsupium*.<sup>4,5</sup> Recent studies suggest that pterostilbene exhibits the hallmark characteristics of an effective

anticancer agent on the basis of its antineoplastic properties in several common malignant tumors. Pterostilbene induces apoptosis in human gastric cancer cells and multidrug resistant leukemia cells by the induction of mitochondrial genes.<sup>6,7</sup> Pterostilbene inhibits metastatic activity in B16 melanoma cells<sup>8</sup> and in invasive and aggressive phenotypes of breast cancer cells.<sup>9</sup> Previous papers also described growth inhibitory effects of pterostilbene on prostate cancer cells,<sup>10–12</sup> however, its molecular mechanism remains inconclusive.

The AMP-activated protein kinase (AMPK) is a major regulator of cellular and organismal energy homeostasis and signal transducer that is regulated by a wide array of metabolic stresses.<sup>13–15</sup> AMPK is activated by a variety of cellular stresses that decrease ATP-consuming metabolic functions (such as protein synthesis, sterol synthesis, and glycogen synthesis) and increase ATP-producing activities (such as glucose uptake, fatty acid oxidation, and mitochondrial biogenesis) to restore energy

Received: April 10, 2012

Revised: June 6, 2012

Accepted: June 6, 2012

Published: June 6, 2012

homeostasis. Thus, AMPK functions as a central metabolic switch that governs glucose and lipid metabolism.<sup>14</sup> Previous studies have revealed an association between polymorphisms in the *PRKAA2* gene (encoding the  $\alpha 2$  subunit of AMPK)<sup>16</sup> and susceptibility to insulin resistance and diabetes in the Japanese population.<sup>17</sup> Interestingly, the same locus correlates with prostate cancer risk,<sup>18</sup> suggesting that AMPK dysregulation may provide a mechanistic link between diabetes and prostate cancer. Therefore, AMPK activators may be beneficial for prostate cancer prevention and treatment.

Fatty acids are important for functions of cells including energy storage, membrane structure, and signal transduction cascades.<sup>19</sup> Cancer cells synthesize large amounts of de novo fatty acids and cholesterol and benefit from this increased fatty acid synthesis in terms of proliferation, survival, and drug resistance. Past studies showed that fatty acid synthesis is significantly elevated in a variety of cancer cells.<sup>20</sup> Fatty acid synthase (FASN) is a key enzyme for lipogenesis. Numerous studies have shown that FASN is often highly expressed in human cancers.<sup>20</sup> Nearly all prostate cancer expresses high levels of the enzyme FASN, suggesting that exacerbated lipogenesis affects multiple key aspects of tumor cell biology.

It becomes increasingly clear that obesity can cause perturbations in metabolic pathways that contribute to the pathogenesis and progression of prostate cancer. Hence, the use of AMPK activators and inhibitors of key lipogenic enzymes may represent a promising therapeutic strategy for prostate cancer. In this study, we hypothesized whether pterostilbene up-regulated AMPK activity and suppressed cell proliferation in p53 positive and negative prostate cancer cells.

## MATERIALS AND METHODS

**Chemicals.** Pterostilbene, compound C, Oil Red O staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), and antibodies for  $\beta$ -actin were purchased from Sigma (St. Louis, MO, USA). Antibodies for FASN, phospho-acetyl-CoA carboxylase (ACC) (Ser 79), phospho-AMPK (Thr 172), AMPK, caspase-3, caspase-9, PARP, p21, and p53 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for mouse and rabbit conjugated with horseradish peroxidase were purchased from Chemicon (Temecula, CA, USA). Lipofectamin 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Immobilon Western Chemiluminescent HRP Substrate was from Millipore Corp. (Billerica, MA, USA). The other chemicals and reagents were from Sigma.

**Cell Culture.** Human prostate cells, LNCaP (p53 wild type) and PC3 (p53 null) cell lines, were grown in RPMI-1640 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

**MTT Assay.** Cells ( $1 \times 10^4$ ) were seeded on the 24-well cell culture cluster overnight and then treated with different concentrations of pterostilbene and incubated for 48 h. Next, 40  $\mu$ L of MTT (stock concentration = 2 mg/mL, Sigma Chemical Co.) was added to each well (the volume of every well was 500  $\mu$ L) and then incubated for 2 h at 37 °C. MTT–formazan crystals will be formed, and then added 250  $\mu$ L of dimethyl sulfoxide (DMSO) is used to dissolve the crystals. Finally, the absorbance at OD 550 nm was determined by the enzyme-linked immunosorbent assay (ELISA) reader.

**Western Blot.** Cells ( $1 \times 10^6$ ) were twice washed with PBS and then the gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM PMSF, 10  $\mu$ g/mL leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100 mM  $\beta$ -glycerophosphate, 20 mM Tris-HCl, 137 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 10  $\mu$ g/mL aprotinin, adjunct pH 7.9) was added. To take the suspension solution after the cell lysates

were centrifuged, the Bio-Rad protein assay kit (Bio-Rad Laboratories) was used to determine the protein content. Fifty micrograms of proteins was used to resolve by SDS-PAGE and transferred to a PVDF membrane (polyvinylidene fluoride transfer membrane) (BioTrace, U.K.). Membrane was blocked by blocking buffer (nonfat milk (5%), NaN<sub>3</sub> (0.2%), and Tween 20 (0.2%, v/v) in TBS). Then the PVDF membrane was incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 dilution, Roche Applied Science, Indianapolis, IN, USA). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL, USA). The intensity of the bands was scanned and quantified with a Phosphor-Image system.

**Oil Red O Staining.** Cells ( $2 \times 10^5$ ) were seeded on the 6-well cell culture cluster overnight. Cells were treated with pterostilbene, AICAR, or compound C and then incubated for 48 h. Cells were washed with PBS twice and fixed with 10% formaldehyde for 1 h. Then they were washed with PBS and 50% isopropanol. Next, Oil Red O staining working buffer (stock solution, 3 mg/mL in isopropanol; working solution, 60% Oil Red O stock solution) was added for 1 h, and then the mixture was washed with PBS and 70% ethanol. Finally, 250  $\mu$ L of isopropanol was added to dissolve the Oil Red O, and the absorbance at OD 510 nm was detected by the ELISA reader.

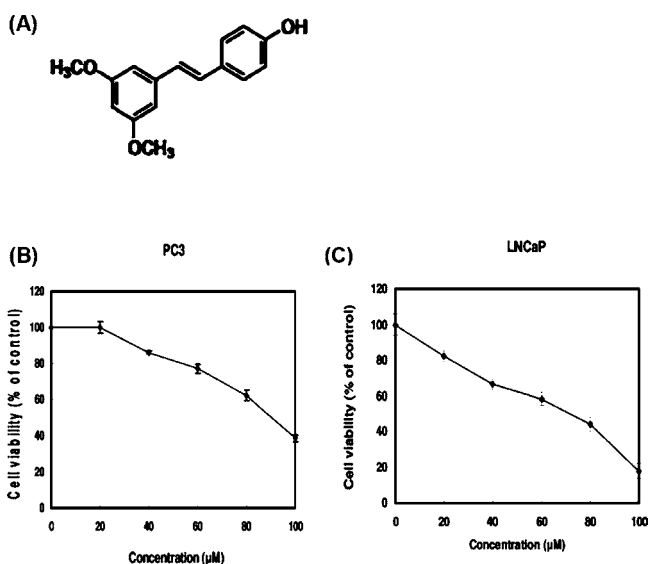
**Short Hairpin RNA.** RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, supported by the National Research Program for Genomic Medicine Grants of NSC (NSC 97-3112-B-001-016). Short hairpin RNAs (shRNAs) were designed to target specific sequences of human AMPK (clone ID, TRCN0000010436; target sequence, 5'-GCATAATAAGTC ACAGC-CAAA-3'). One day before transfection, cells were seeded without antibiotics at the density of 30–40%. Twenty nanomolar AMPK shRNAs was transfected into cells by lipofectamine 2000 (Invitrogen). Cells were incubated for an additional 24 h, and pterostilbene was added as previously described. The cell lysates were analyzed by Western blot as previously described.

**Cell-Cycle Analysis.** Cells ( $5 \times 10^5$ ) were cultured in a 6 mm cell culture dish and treated with 80  $\mu$ M pterostilbene for the indicated duration. Then cells were harvested in a 15 mL tube, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100% ethanol at –20 °C overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5  $\mu$ g/mL RNase), and incubated at room temperature for 30 min. Then 1 mL of PI solution (50  $\mu$ g/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the PI–DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA, USA).

**Statistical Analysis.** All values are expressed as the mean  $\pm$  SD. Each value is the mean of at least three separate experiments in each group; Student's *t* test was used for statistical comparison. Asterisks indicate the values are significantly different from the control (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

## RESULTS

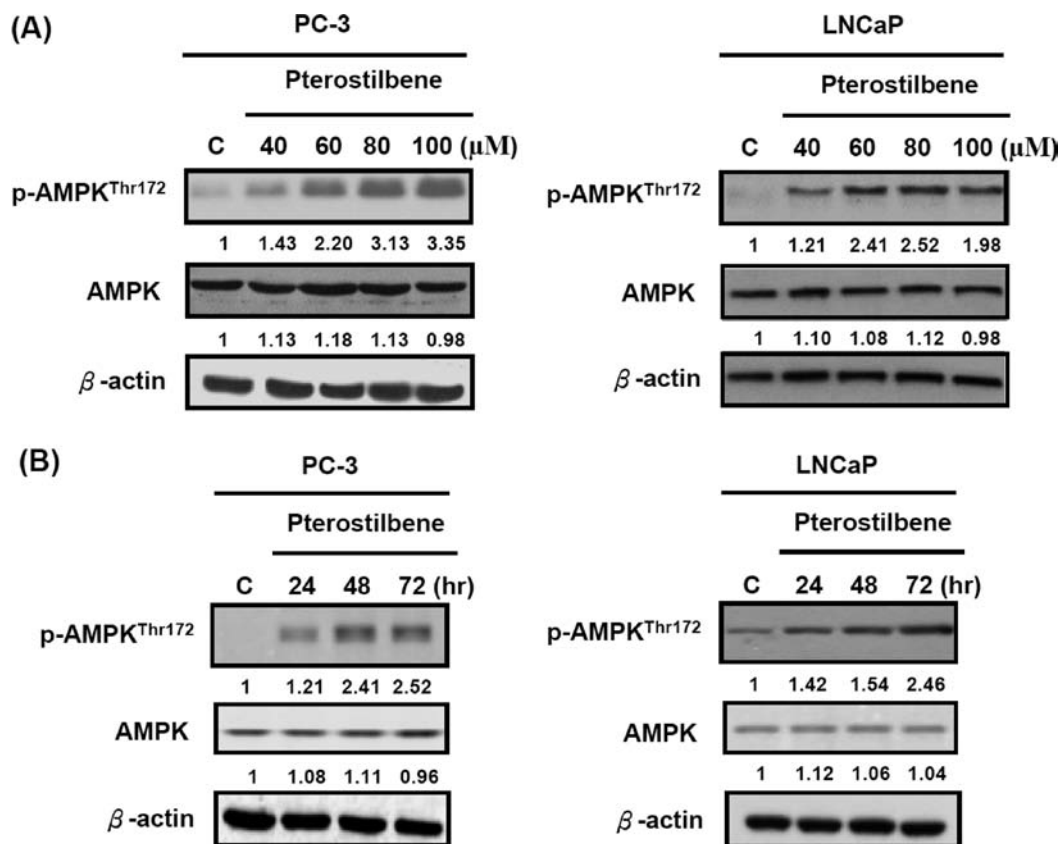
**Pterostilbene Up-regulates AMPK Activity in p53 Positive and Negative Prostate Cancer Cells.** To investigate the bioactivity of pterostilbene in p53 positive and negative prostate cells, we treated PC3 cells (p53 null) and LNCaP cells (p53 wild type) with different concentrations of pterostilbene (Figure 1A) for 48 h and assessed cell proliferation by MTT assay. As shown in Figure 1, the growth of PC3 cells (Figure 1B) and LNCaP cells (Figure 1C) was inhibited by pterostilbene in a dose-dependent manner. We next examined whether the antiproliferative effects of pterostilbene involved the up-regulation of AMPK activity in p53 positive and negative prostate cancer cells. PC3 cells and LNCaP cells were treated with different concentrations of



**Figure 1.** Proliferation-inhibitory effect of both p53 positive and negative prostate cancer cells: (A) structure of pterostilbene; treatment of (B) PC3 and (C) LNCaP cells with various concentrations of pterostilbene at 37 °C for 48 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without pterostilbene as 100%. This experiment was repeated three times. Bar represent the SD.

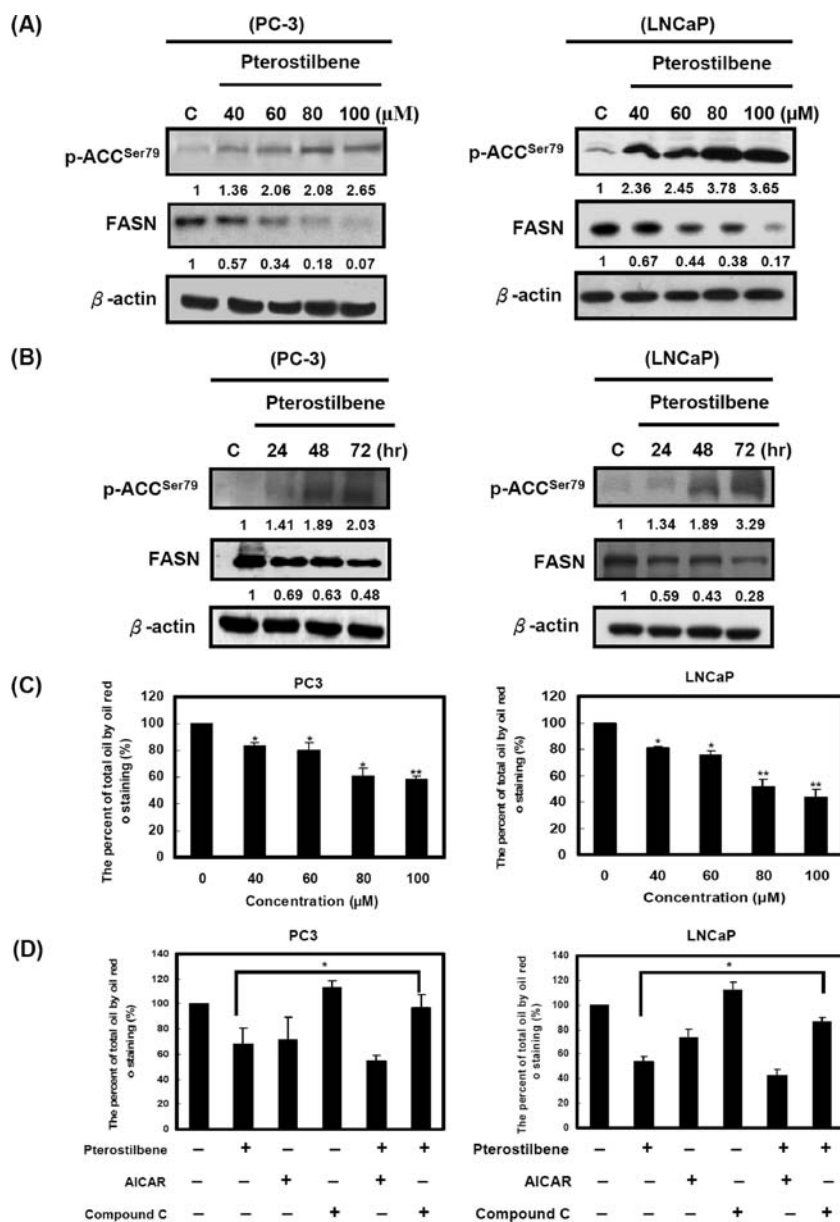
pterostilbene for 48 h. Western blot analysis indicated that pterostilbene stimulated AMPK phosphorylation in a dose-dependent manner (Figure 2A). To address this time-dependent effect, we treated PC3 cells and LNCaP cells with 80  $\mu\text{M}$  pterostilbene for various periods of time. Western blot analysis indicated that pterostilbene stimulated AMPK phosphorylation in a time-dependent manner (Figure 2B). Those results showed that pterostilbene up-regulated AMPK activity and suppressed cell proliferation in p53 positive and negative prostate cells.

**Pterostilbene Decreases Lipid Synthesis by Decreasing FASN Expression and Inhibiting ACC Activity.** The activities of FASN and ACC were known to be negatively regulated by AMPK.<sup>21</sup> We next examined whether pterostilbene decreased lipid synthesis by decreasing FASN expression and inhibiting ACC activity. PC3 cells and LNCaP cells were treated with different concentrations of pterostilbene for 48 h. Western blot analysis indicated that pterostilbene decreased the protein levels of FASN and increased ACC phosphorylation in a dose-dependent manner (Figure 3A). To address this time-dependent effect, we treated PC3 cells and LNCaP cells with 80  $\mu\text{M}$  pterostilbene for various periods of time. Western blot analysis indicated that pterostilbene decreased the protein levels of FASN and increased ACC phosphorylation in a time-dependent manner (Figure 3B). To examine whether pterostilbene influences total fatty acid content, we treated PC3 and LNCaP cells with different



**Figure 2.** Pterostilbene activates AMPK in both p53 positive and negative prostate cancer cells. (A) PC3 and LNCaP cells were treated with DMSO (control) or different concentrations of pterostilbene for 48 h. (B) PC3 and LNCaP cells were treated with 80  $\mu\text{M}$  pterostilbene for the indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phosphorylated AMPK (Thr 172), AMPK, and  $\beta$ -actin. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

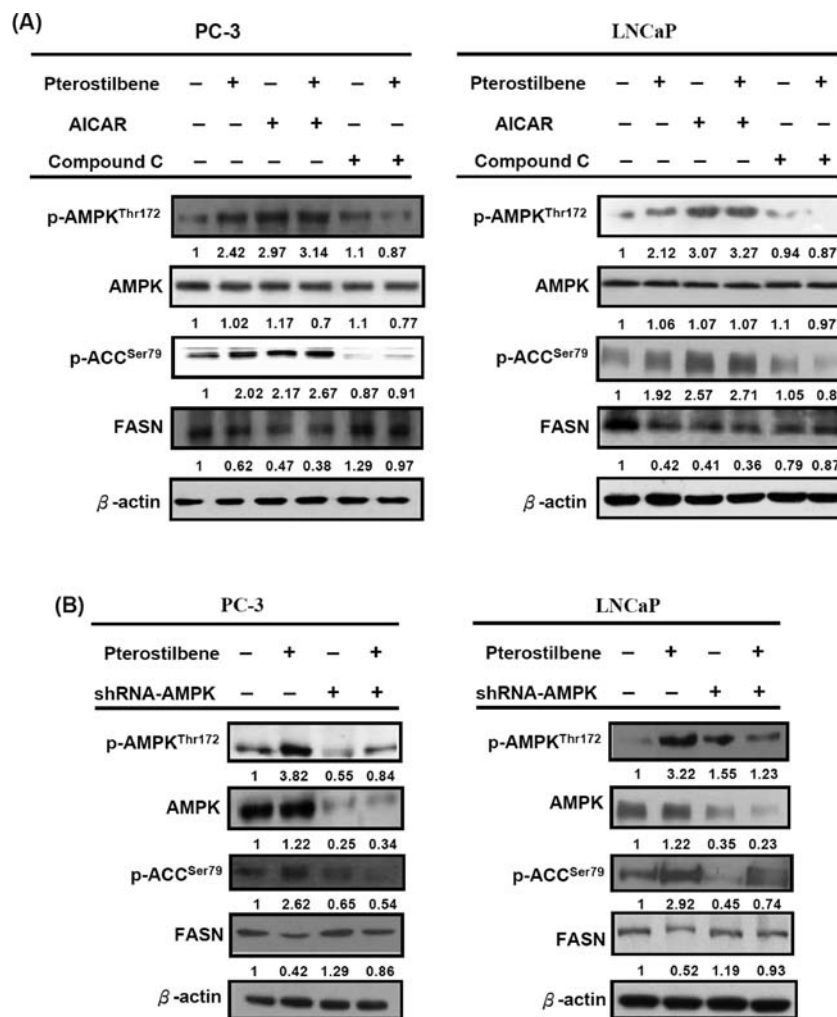




**Figure 3.** Pterostilbene decreases the activity of fatty acid synthesis by inhibiting the expression of FASN and the activity of ACC. (A) PC3 and LNCaP cells were treated with DMSO (control) or different concentrations of pterostilbene for 48 h. (B) PC3 and LNCaP cells were treated with 80  $\mu$ M pterostilbene for the indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against FASN, phospho-ACC (Ser79), and  $\beta$ -actin. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated. (C) PC3 and LNCaP cells were treated with DMSO (control) or different concentrations of pterostilbene for 48 h. (D) PC3 and LNCaP cells were treated with 15  $\mu$ M compound C or 500  $\mu$ M AICAR in the absence or presence of 80  $\mu$ M pterostilbene for 48 h. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without pterostilbene as 100%. This experiment was repeated three times. Bar represent the SD. Student's *t* test was used for a statistical comparison. Asterisks indicate that the values are significantly different from the control (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

concentrations of pterostilbene for 48 h, and examined the total lipids by Oil Red O staining. Oil Red O staining analysis indicated that pterostilbene decreased the total fatty acid content in a dose-dependent manner (Figure 3C). This implied that intracellular lipid content could be reduced significantly by treatment with pterostilbene. To further study the effect of AMPK in regulating the total fatty acid content, compound C, an AMPK inhibitor, was added in the absence or presence of pterostilbene. Results showed the lipids content was decreased in PC3 and LNCaP cells treated with pterostilbene but increased in cells treated with pterostilbene and compound C,

showing that the lipid synthesis was restored when compound C was added (Figure 3D). Moreover, the hypolipidemic potential of pterostilbene was similar to that of AICAR, a known AMPK activator, in PC3 and LNCaP cells (Figure 3D). We also tested for the effect of cotreatment with pterostilbene (80  $\mu$ M) and AICAR (500  $\mu$ M) on total fatty acid content. In the copresence of pterostilbene and AICAR, the total fatty acid content was decreased more potently than by treatment with pterostilbene or AICAR alone in PC3 and LNCaP cells (Figure 3D). These results might suggest that pterostilbene decreased



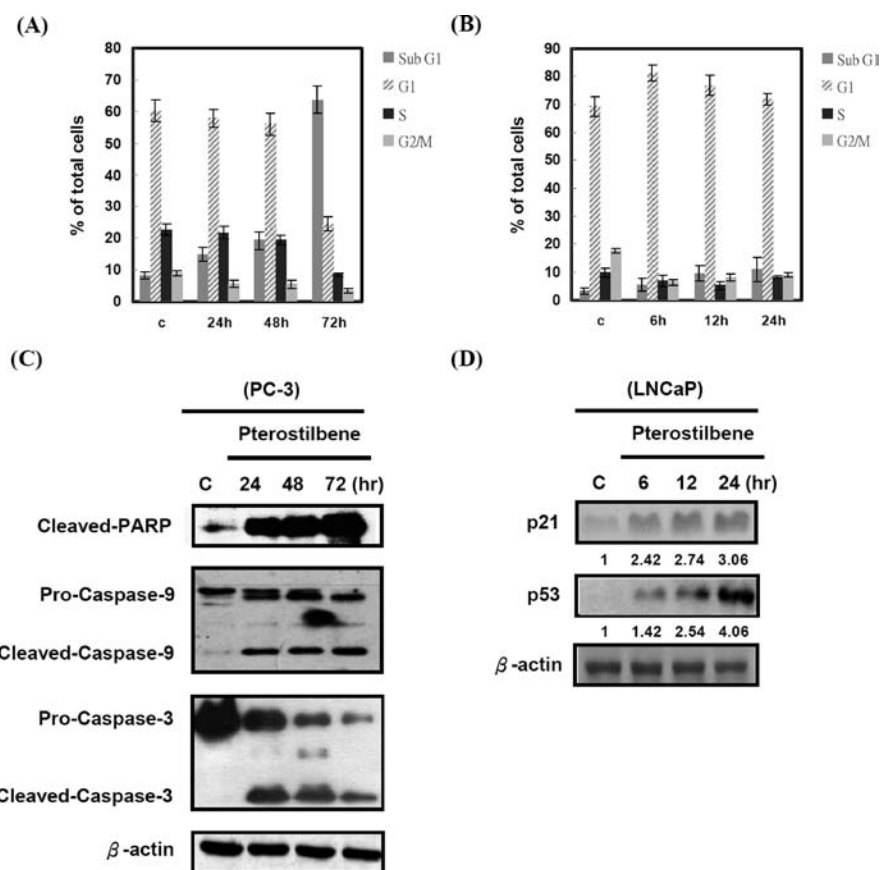
**Figure 4.** Pterostilbene decreases the activity of fatty acid synthesis via activation of AMPK. (A) PC3 and LNCaP cells were treated with 15  $\mu$ M compound C or 500  $\mu$ M AICAR in the absence or presence of 80  $\mu$ M pterostilbene for 48 h. (B) PC3 and LNCaP cells were transfected with 50 nmol/L AMPK $\alpha$ 1-shRNA using Oligofectamine. Twenty-four hours after transfection, cells were treated with 80  $\mu$ M pterostilbene for 48 h. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phosphorylated AMPK (Thr 172), AMPK, phospho-ACC (Ser79), FASN, and  $\beta$ -actin. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

the total lipid content in p53 positive and negative prostate cancer cells by activating AMPK.

**Pterostilbene Suppresses Lipid Synthesis by Activating AMPK.** To further study the effect of AMPK in regulating the activity of fatty acid synthesis enzymes, we added compound C in the absence or presence of pterostilbene. After the treatment with pterostilbene, the protein levels of FASN were decreased and phospho-ACC was increased. However, the activities of enzymes of fatty acid synthesis were restored in the presence of compound C in PC3 and LNCaP (Figure 4A) cells. We also tested for the effect of cotreatment with pterostilbene (80  $\mu$ M) and AICAR (500  $\mu$ M) in regulating the activity of fatty acid synthesis enzymes. In the copresence of pterostilbene and AICAR, the protein levels of FASN were decreased and phospho-ACC was increased more potently than by treatment with pterostilbene or AICAR alone in PC3 and LNCaP cells (Figure 4A). In addition, we also treated AMPK shRNA to silence the AMPK. The AMPK shRNA rescued cells from the inhibitory effect of pterostilbene. The activities of fatty acid synthesis enzymes were not inhibited by pterostilbene when transfected with AMPK shRNA in PC3

and LNCaP (Figure 4B) cells. These results demonstrated that pterostilbene inhibited the activity of fatty acid synthesis enzymes and decreased total lipid content through the activation of AMPK pathway.

**Pterostilbene Induces LNCaP Growth Arrest, but Induces PC3 Apoptosis.** The resolution between apoptosis and growth arrest following FASN inhibition is greatly influenced by p53 status.<sup>21,22</sup> To examine whether pterostilbene produces both cytostatic and cytotoxic effects modulated by p53, we used PI stain to measure flow cytometrically. Cell cycle of LNCaP (Figure 5B) arrested in G1 phase; in contrast, PC3 cells underwent apoptosis (Figure 5A). Moreover, we examined the expression of G1-related cell-cycle control proteins and apoptosis-related proteins on Western blot analysis. PC3 and LNCaP cells were treated with 80  $\mu$ M pterostilbene with the indicated durations. Western blot analysis indicated that treatment with 80  $\mu$ M pterostilbene of PC3 cells resulted in a clear apoptosis within 72 h, showing cleavages for PARP, caspase-3, and caspase-9 in Western blot analyses (Figure 5C). After 6 h of pterostilbene treatment, we could find increased levels of p21 and p53 in LNCaP cells (Figure 5D). These



**Figure 5.** Pterostilbene induces cell-cycle arrest in p53 positive cells, but apoptosis in p53 negative cells. (A) PC3 cells and (B) LNCaP cells were treated with 80  $\mu$ M pterostilbene for the indicated duration and analyzed for PI-stained DNA content by flow cytometry. The indicated percentages are the mean of three independent experiments, each in duplicate. Bars represent the SD. (C) PC3 cells were treated with vehicle (DMSO) or pterostilbene (80  $\mu$ M) for the indicated time. Cells were then harvested and lysed for the detection of cleaved PARP, caspase-9, caspase-3, and  $\beta$ -actin protein expression. (D) LNCaP cells were treated with vehicle (DMSO) or pterostilbene (80  $\mu$ M) for the indicated time. Cells were then harvested and lysed for the detection of p21, p53, and  $\beta$ -actin protein expression. Western blot data presented are representative of those obtained in at least three separate experiments. Immunoblots were quantified, and relative expression to control is indicated.

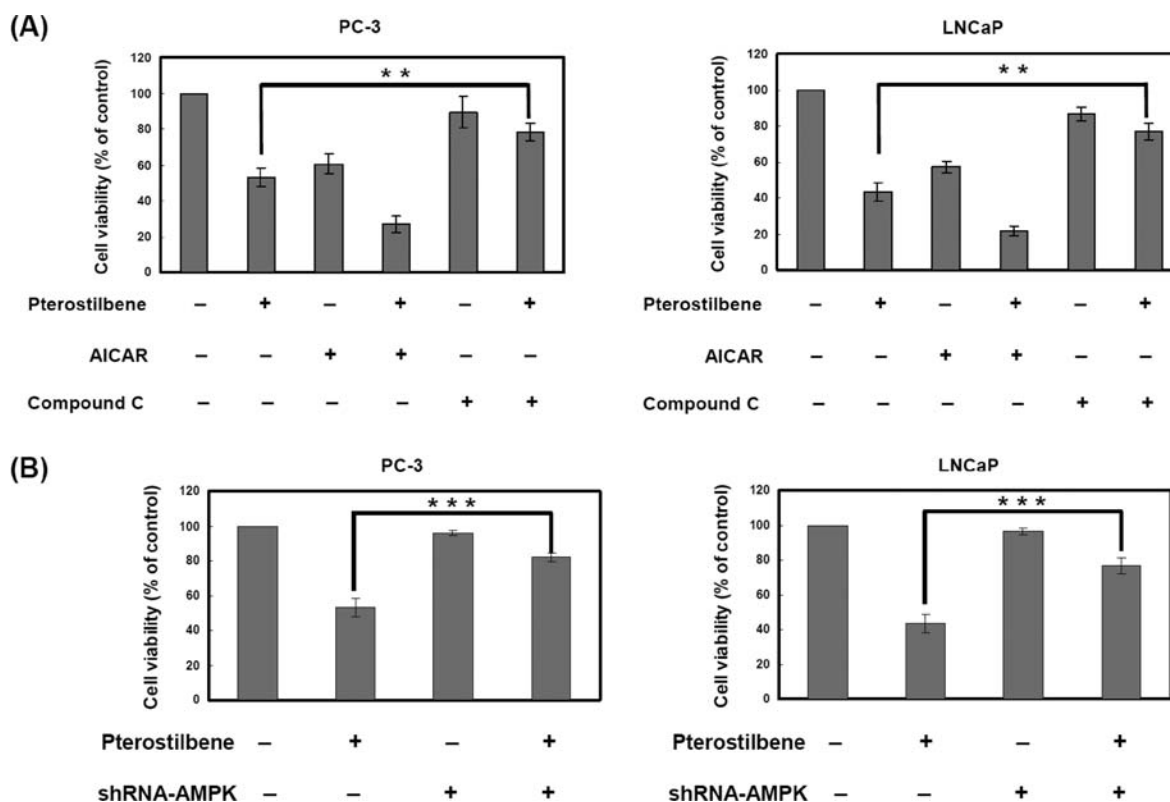
results demonstrated that pterostilbene, an AMPK activator, was more effective at initiating apoptosis in tumor cells with nonfunctioning p53, whereas cells with intact p53 function tended to exhibit cytostatic responses.

**Pterostilbene Inhibits Prostate Cancer Cell Proliferation by Activating AMPK.** To further study the effect of AMPK in regulating prostate cancer cell proliferation, we added compound C, in the absence or presence of pterostilbene. After the treatment with pterostilbene, cell viability was decreased. However, cell viability was restored in the presence of compound C in PC3 and LNCaP (Figure 6A) cells. We also tested the effect of cotreatment with pterostilbene (80  $\mu$ M) and AICAR (500  $\mu$ M) in regulating prostate cancer cell proliferation. In the copresence of pterostilbene and AICAR, cell viability was decreased more potently than by treatment with pterostilbene or AICAR alone in PC3 and LNCaP cells (Figure 6A). In addition, we also treated AMPK shRNA to silence the AMPK. The AMPK shRNA rescued cells from the inhibitory effect of pterostilbene. Cell viability was not inhibited by pterostilbene when transfected with AMPK shRNA in PC3 and LNCaP (Figure 6B) cells. These results demonstrated that pterostilbene inhibited prostate cancer cell proliferation through activation of the AMPK pathway.

## DISCUSSION

Even though the association between a high-fat diet and prostate cancer risk is controversial, it is becoming increasingly evident that obesity can cause perturbations in metabolic pathways that contribute to the pathogenesis and progression of prostate cancer.<sup>23</sup> Key lipogenic alterations that commonly occur in prostate cancer include overexpression of the FASN and deregulation of the AMPK. Recent studies have focused on the potential of targeting metabolic pathways that may be altered during prostate tumorigenesis and progression.<sup>23</sup> Several small molecule inhibitors of FASN have now been described or are in development for therapeutic use; in addition, drugs that directly or indirectly induce AMPK activation have potential benefit in prostate cancer prevention and treatment.<sup>24–26</sup> Here, our studies showed that pterostilbene induced AMPK activation and inhibited key lipogenic enzymes in p53 positive and negative prostate cancer cells. Hence, the use of AMPK activators and inhibitors of key lipogenic enzymes may represent a promising therapeutic strategy for prostate cancer.

AMPK, an energy-sensing serine/threonine kinase, is a major regulator of cellular and organismal energy homeostasis that coordinates multiple metabolic pathways to balance energy supply and demand, indicating its fundamental role in cellular regulation. Links between AMPK and cancer can be made at



**Figure 6.** Pterostilbene inhibits both p53 positive and negative prostate cancer cell proliferation via activation of AMPK. (A) PC3 and LNCaP cells were treated with 15  $\mu$ M compound C or 500  $\mu$ M AICAR in the absence or presence of 80  $\mu$ M pterostilbene for 48 h. (B) PC3 and LNCaP cells were transfected with 50 nmol/L AMPK $\alpha$ 1-shRNA using Oligofectamine. Twenty-four hours after transfection, cells were treated with 80  $\mu$ M pterostilbene for 48 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without pterostilbene as 100%. This experiment was repeated three times. Bars represent the SD. Student's *t* test was used for statistical comparison. Asterisks indicate that the values are significantly different from the control (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

both the level of the organism and the molecular level. Decreased AMPK activation is implicated in human metabolic disorders associated with increased cancer risk. Drugs that ameliorate metabolic syndrome conditions through AMPK activation may be beneficial for prostate cancer prevention and treatment. The tumor prevention effect by natural products such as berberine has been partly attributed to AMPK activation. Berberine and its more biologically available derivative, dihydroberberine, improve insulin sensitivity in rodent models of insulin resistance through the activation of AMPK by an indirect mechanism similar to that of metformin and thiazolidinediones (TZDs).<sup>27</sup> A number of other well-known chemopreventive agents such as resveratrol, EGCG, genistein, and selenium are competent to activate AMPK. However, the contribution of AMPK to their preventive actions is still unknown.<sup>28</sup>

De novo fatty acid and sterol syntheses are common features of both primary and advanced prostate cancer.<sup>29</sup> FASN and ACC are responsible for the synthesis of malonyl-CoA, palmitate, and mevalonate (the precursor of cholesterol), respectively, and their role in the pathogenesis and progression of prostate cancer is well established.<sup>20</sup> Pharmacologic inhibition of FASN has been used to study the loss of FASN function in tumor cells. Small-molecule FASN inhibitors have shown promising preclinical results. Recent studies have reported that EGCG, the main polyphenol of green tea, and other dietary flavonoids (such as luteolin, quercetin, and kaempferol) inhibit FASN, induce apoptosis of several tumor cell lines in vitro, and reduce the size of mammary tumors in

animal models.<sup>30</sup> So far, the use of FASN inhibitors as systemic drugs has been hampered by pharmacologic limitations and side effects (weight loss). Even though most attention on the role of endogenous fatty acid metabolism in prostate cancer has been directed toward the overexpression of FASN, in recent years other key lipid enzymes such as HMG-CoA reductase and ACC have been indicated as potential targets for prostate cancer.<sup>31</sup> Aggressive cancer cells have a high energy-demanding anabolic activity, and that anabolism is intrinsically regulated by the cellular energy supply. This provided evidence for an AMPK-regulated link between energy status, tumor-associated lipogenic metabolism, and the malignant phenotype.<sup>32</sup> Pharmacologically inducing a "calorie-restriction status" in tumor cells would result in AMPK-induced ACC phosphorylation, FASN down-regulation, and a marked decrease of endogenous lipogenesis, and cancer cells would stop proliferating and lose their invasive properties and their ability to form colonies. Here, we have found that pterostilbene has beneficial activities similar to those associated with low-energy mimickers, and these might be related to uncharacterized direct actions of pterostilbene on cancer cells. AMPK is activated by pterostilbene and is required for antiproliferative effects of pterostilbene in p53 positive and negative prostate cancer cells.

Recently, it has been suggested that cells require specific extracellular signals to license their ability to take up nutrients for cell division. This implies that there exist mechanisms by which cells coordinate proliferation relative to their ability to take up specific extracellular metabolites. Recent study also found that induction of AMPK activity results in cell-cycle



arrest at levels of extracellular glucose that are still capable of supporting the proliferative expansion of cells. The ability of AMPK to induce cell-cycle arrest is dependent on p53.<sup>33</sup> The ability of AMPK to promote senescence or to inhibit cell proliferation in response to energy starvation has been interpreted as a check point that couples glucose availability to the progression of the cell cycle; it was implied that the activation of AMPK might promote the conservation of the remaining energy to support the survival and physiological functions of the cell during cell-cycle arrest. The resolution between apoptosis and growth arrest after AMPK activation is clearly influenced by p53 function. Our results indicated that pterostilbene inhibited the proliferation of cells via the activation of AMPK. LNCaP cells have a normal functional p53, whereas PC3 cells are p53 null. In LNCaP cells, pterostilbene treatment inhibited the progression of cell cycle in the G1 phase. Pterostilbene increased the expression level of p53 and subsequently enhanced the expression level of p21, resulting in cell-cycle arrest in LNCaP cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after AMPK activation. Moreover, our study also confirmed a recent study postulating that pterostilbene was found to cause apoptosis in PC3 cells.<sup>12</sup>

Pterostilbene and resveratrol have similar pharmacologic properties; however, pterostilbene has several advantages. Pterostilbene contains two methoxy groups and one hydroxy group, whereas resveratrol has three hydroxy groups. The two methoxy groups cause pterostilbene to be more lipophilic, increasing oral absorption and rendering a higher potential for cellular uptake. When administered orally, pterostilbene shows 95% bioavailability, whereas resveratrol has only 20% bioavailability.<sup>34</sup> The half-life of pterostilbene in serum is also 7 times longer than resveratrol, 105 versus 14 min.<sup>35</sup> Moreover, a recent study has reported that dietary administration of high doses of pterostilbene to mice is not toxic.<sup>36</sup> Further efforts, including a phase I study, will be needed to adequately define pterostilbene's safety and dosing in humans. Taken together, our study suggests that pterostilbene may be useful as a prostate cancer chemopreventive agent.

## AUTHOR INFORMATION

### Corresponding Author

\* (J.-Y.W.) Postal address: Department of Chemical Engineering, Institute of Biotechnology and Chemical Engineering, I-Shou University, No. 1, Sec. 1, Syuecheng Road, Dashu District, Kaohsiung City 84001, Taiwan, ROC. Phone: (886)-7-6577711. E-mail: wujy@isu.edu.tw. (T.-D.W.) Postal address: Department of Biological Science and Technology, College of Life Sciences, China Medical University, No. 91 Hsueh-Shih Road, Taichung, Taiwan 40402, ROC. Phone: (886)-4-2205-3366, ext. 2509. Fax: (886)-4-2207-0465. E-mail: tdway@mail.cmu.edu.tw.

### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FASN, fatty acid synthase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

## REFERENCES

- (1) Jemal, A.; Siegel, R.; Ward, E.; Murray, T.; Xu, J.; Thun, M. J. Cancer statistics 2007. *CA Cancer J. Clin.* **2007**, *57*, 43–66.
- (2) Hsing, A. W.; Devesa, S. S. Trends and patterns of prostate cancer: what do they suggest? *Epidemiol. Rev.* **2001**, *23*, 3–13.
- (3) Giovannucci, E.; Rimm, E. B.; Colditz, G. A.; Stampfer, M. J.; Ascherio, A.; Chute, C. C.; Willett, W. C. A prospective study of dietary fat and risk of prostate cancer. *J. Natl. Cancer Inst.* **1993**, *85*, 1571–1579.
- (4) Roupe, K. A.; Remsberg, C. M.; Yáñez, J. A.; Davies, N. M. Pharmacometrics of stilbenes: segueing towards the clinic. *Curr. Clin. Pharmacol.* **2006**, *1*, 81–101.
- (5) Lin, H. S.; Yue, B. D.; Ho, P. C. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. *Biomed. Chromatogr.* **2009**, *23*, 1308–1315.
- (6) Pan, M. H.; Chang, Y. H.; Badmaev, V.; Nagabhushanam, K.; Ho, C. T. Pterostilbene induces apoptosis and cell cycle arrest in human gastric carcinoma cells. *J. Agric. Food Chem.* **2007**, *55*, 7777–7785.
- (7) Tolomeo, M.; Grimaudo, S.; Cristina, A. D.; Roberti, M.; Pizzirani, D.; Meli, M.; Dusochet, L.; Gebbia, N.; Abbadessa, V.; Crosta, L.; Barucchello, R.; Grisolia, G.; Invidiata, F.; Simoni, D. Pterostilbene and 3'-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 1709–1726.
- (8) Ferrer, P.; Asensi, M.; Ramon, S.; Ortega, A.; Benlloch, M.; Obrador, E.; Varea, M. T.; Asensio, G.; Jorda, L.; Estrela, J. M. Association between pterostilbene and quercetin inhibits metastatic activity of B16 melanoma. *Neoplasia* **2005**, *7*, 37–47.
- (9) Pan, M. H.; Lin, Y. T.; Lin, C. Li.; Wei, C. S.; Ho, C. T.; Chen, W. J. Suppression of heregulin- $\beta$ 1/HER2-modulated invasive and aggressive phenotype of breast carcinoma by pterostilbene via inhibition of matrix metalloproteinase-9, p38 kinase cascade and Akt activation. *Evidence-Based Complement. Alternat. Med.* **2011**, article ID 562187.
- (10) Horvath, Z.; Marihart-Fazekas, S.; Saiko, P.; Grusch, M.; Ozsüy, M.; Harik, M.; Handler, N.; Erker, T.; Jaeger, W.; Fritzer-Szekeres, M.; Djavan, B.; Szekeres, T. Novel resveratrol derivatives induce apoptosis and cause cell cycle arrest in prostate cancer cell lines. *Anticancer Res.* **2007**, *27*, 3459–3464.
- (11) Wang, T. T.; Schoene, N. W.; Kim, Y. S.; Mizuno, C. S.; Rimando, A. M. Differential effects of resveratrol and its naturally occurring methylether analogs on cell cycle and apoptosis in human androgen-responsive LNCaP cancer cells. *Mol. Nutr. Food Res.* **2010**, *54*, 335–344.
- (12) Chakraborty, A.; Gupta, N.; Ghosh, K.; Roy, P. In vitro evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from *Pterocarpus marsupium*. *Toxicol. In Vitro* **2010**, *24*, 1215–1228.
- (13) Hedbacker, K.; Carlson, M. SNF1/AMPK pathways in yeast. *Front. Biosci.* **2008**, *13*, 2408–2420.
- (14) Shaw, R. J.; Kosmatka, M.; Bardeesy, N.; Hurley, R. L.; Witters, L. A.; DePinho, R. A.; Cantley, L. C. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 3329–3335.
- (15) Woods, A.; Dickerson, K.; Heath, R.; Hong, S. P.; Momcilovic, M.; Johnstone, S. R.; Carlson, M.; Carling, D. Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase- $\beta$  acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* **2005**, *2*, 21–33.
- (16) Viollet, B.; Andreelli, F.; Jørgensen, S. B.; Perrin, C.; Flamez, D.; Mu, J.; Wojtaszewski, J. F.; Schuit, F. C.; Birnbaum, M.; Richter, E.; Burcelin, R.; Vaulont, S. Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models. *Biochem. Soc. Trans.* **2003**, *31*, 216–219.
- (17) Horikoshi, M.; Hara, K.; Ohashi, J.; Miyake, K.; Tokunaga, K.; Ito, C.; Kasuga, M.; Nagai, R.; Kadowaki, T. A polymorphism in the AMPK $\alpha$ 2 subunit gene is associated with insulin resistance and type 2 diabetes in the Japanese population. *Diabetes* **2006**, *55*, 919–923.



- (18) Matsui, H.; Suzuki, K.; Ohtake, N.; Nakata, S.; Takeuchi, T.; Yamanaka, H.; Inoue, I. Genomewide linkage analysis of familial prostate cancer in the Japanese population. *J. Hum. Genet.* **2004**, *49*, 9–15.
- (19) Kuhajda, F. P. Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. *Nutrition* **2000**, *16*, 202–208.
- (20) Menendez, J. A.; Lupu, R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* **2007**, *7*, 763–777.
- (21) Li, J. N.; Gorospe, M.; Chrest, F. J.; Kumaravel, T. S.; Evans, M. K.; Han, W. F.; Pizer, E. S. Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. *Cancer Res.* **2001**, *61*, 1493–1499.
- (22) Menendez, J. A.; Lupu, R. RNA interferencemediated silencing of the p53 tumor-suppressor protein drastically increases apoptosis after inhibition of endogenous fatty acid metabolism in breast cancer cells. *Int. J. Mol. Med.* **2005**, *15*, 33–40.
- (23) Zadra, G.; Priolo, C.; Patnaik, A.; Loda, M. New strategies in prostate cancer: targeting lipogenic pathways and the energy sensor AMPK. *Clin. Cancer Res.* **2010**, *16*, 3322–3328.
- (24) Kridel, S. J.; Axelrod, F.; Rozenkrantz, N.; Smith, J. W. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. *Cancer Res.* **2004**, *64*, 2070–2075.
- (25) Murtola, T. J.; Visakorpi, T.; Lahtela, J.; Syväälä, H.; Tammela, T. Lj. Statins and prostate cancer prevention: where we are now, and future directions. *Nat. Clin. Pract. Urol.* **2008**, *5*, 376–387.
- (26) Xiang, X.; Saha, A. K.; Wen, R.; Ruderman, N. B.; Luo, Z. AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. *Biochem. Biophys. Res. Commun.* **2004**, *321*, 161–167.
- (27) Turner, N.; Li, J. Y.; Gosby, A.; To, S. W.; Cheng, Z.; Miyoshi, H.; Taketo, M. M.; Cooney, G. J.; Kraegen, E. W.; James, D. E.; Hu, L. H.; Li, J.; Ye, J. M. Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. *Diabetes* **2008**, *57*, 1414–1418.
- (28) Aggarwal, B. B.; Shishodia, S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* **2006**, *71*, 1397–1421.
- (29) Rossi, S.; Graner, E.; Febbo, P.; Weinstein, L.; Bhattacharya, N.; Onody, T.; Buble, G.; Balk, S.; Loda, M. Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol. Cancer Res.* **2003**, *1*, 707–715.
- (30) Lupu, R.; Menendez, J. A. Pharmacological inhibitors of fatty acid synthase (FASN)-catalyzed endogenous fatty acid biogenesis: a new family of anti-cancer agents? *Curr. Pharm. Biotechnol.* **2006**, *7*, 483–493.
- (31) Flavin, R.; Zadra, G.; Loda, M. Metabolic alterations and targeted therapies in prostate cancer. *J. Pathol.* **2011**, *223*, 283–294.
- (32) Swinnen, J. V.; Beckers, A.; Brusselmans, K.; Organe, S.; Segers, J.; Timmermans, L.; Vanderhoydonc, F.; Deboel, L.; Derua, R.; Waelkens, E.; De Schrijver, E.; Van de Sande, T.; Noël, A.; Foulfelle, F.; Verhoeven, G. Mimicry of a cellular low energy status blocks tumor cells anabolism and suppresses the malignant phenotype. *Cancer Res.* **2005**, *65*, 2441–2448.
- (33) Jones, R. G.; Plas, D. R.; Kubek, S.; Buzzai, M.; Mu, J.; Xu, Y.; Birnbaum, M. J.; Thompson, C. B. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell* **2005**, *18*, 283–293.
- (34) Lin, H. S.; Yue, B. D.; Ho, P. C. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. *Biomed. Chromatogr.* **2009**, *23*, 1308–1315.
- (35) Remsberg, C. M.; Yáñez, J. A.; Ohgami, Y.; Vega-Villa, K. R.; Rimando, A. M.; Davies, N. M. Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. *Phytother. Res.* **2008**, *22*, 169–179.
- (36) Ruiz, M. J.; Fernández, M.; Picó, Y.; Mañes, J.; Asensi, M.; Carda, C.; Asensio, G.; Estrela, J. M. Dietary administration of high doses of pterostilbene and quercetin to mice is not toxic. *J. Agric. Food Chem.* **2009**, *57*, 3180–3186.