Anticancer Properties of Pomolic Acid-Induced AMP-Activated Protein Kinase Activation in MCF7 Human Breast Cancer Cells

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AMP-activated protein kinase (AMPK) is a sensor of cellular energy status found in all eukaryotes. Recent studies indicate that AMPK activation strongly suppresses cell proliferation in tumor cells, which requires high rates of protein synthesis and de novo fatty acid synthesis for their rapid growth. Pomolic acid (PA) has been previously described as being active in inhibiting the growth of cancer cells. In this study, we investigated PA activated AMPK, and this activity was related to proliferation and apoptosis in MCF7 breast cancer cells. PA inhibited cell proliferation and induced sub-G1 arrest, elevating the mRNA levels of the apoptotic genes p53 and p21. PA activated caspase-3, -9, and poly(ADP-ribose) polymerase, and this effect was inhibited by z-VAD-fmk. AMPK activation was increased by treating cells with PA, inactivated by treating cells with a compound C, and co-treatment consisting of PA and aminomimidazole carboxamide ribonucleotide (AICAR) synergistically activated AMPK. These anti-cancer potentials of PA were accompanied by effects on de novo fatty acid synthesis as shown by the decreased expression of fatty acid synthase, and decreased acetyl-CoA carboxylase activation and incorporation of [3H]acetyl-CoA into fatty acids. In addition, PA inhibited key enzymes involved in protein synthesis such as mammalian target of rapamycin (mTOR), 70 kDa ribosomal protein S6 kinase (p70S6K), and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). These results suggest that PA exerts anti-cancer properties through the modulation of AMPK pathways and its value as an anti-cancer agent in breast cancer therapy.

Key words AMP-activated protein kinase; pomolic acid; breast cancer

Bioactive natural products provide structures that will become major sources of novel agents with pharmacological promise.1) Among the well known anti-cancer drugs are the so-called vinca alkaloids (vinblastin and vincristine), isolated from Catharanthus roseus, and the podophyllotoxins (etoposide and teniposide), which are semisynthetic derivatives of epipodophyllotoxin, extracted from Podophyllum peltatum.2) The anti-tumoral activities of certain di- and tri-terpenes from the bark of Taxus brevifolia, has provided a major renewable natural source of anti-cancer drugs which have displayed activity against several cancers.3) Pomolic acid (PA), a pentacyclic triterpene, was highly effective in inhibiting the growth of leukemia cell lines.4) Apart from its capacity to induce apoptosis, the mechanisms of PA activity are presently unclear. As part of an evaluation of potential of new anti-cancer compounds, this study investigated the molecular pathways of PA-induced cell death, including apoptosis.

AMP-activated protein kinase (AMPK), an evolutionarily conserved fuel-sensing enzyme, is a heterotrimeric serine/threonine protein kinase that is composed of a catalytic α-subunit and regulatory β- and γ-subunits. AMPK activity is allosterically regulated by AMP and through phosphorylation at Thr172 in the activation loop of the α-subunit.5) AMPK acts to maintain the intracellular AMP/ATP ratio, repress pathways that consume energy, and promote ATP-producing catabolic pathways by phosphorylation of downstream targets.6) In addition, AMPK activation stimulates fatty acid oxidation, enhances insulin sensitivity, alleviates hyperglycemia and hyperlipidemia, and inhibits pro-inflammatory changes.7) Thus, AMPK is a well-received therapeutic target for metabolic syndrome and Type 2 diabetes. Recent studies indicate that AMPK plays a role in linking metabolic syndrome and cancer.8) In keeping with in vitro studies, recent epidemiological studies indicate that the incidence of cancer is reduced in Type 2 diabetics treated with metformin, an AMPK activator.9) Observations that AMPK activators such as metformin and adiponectin have antiproliferative activity have generated interest in possible clinical applications.10) Pharmacological agents that are used in the treatment of type 2 diabetes, including thiazolidinediones and metformin, have been shown to activate AMPK. When activated, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), and diminishes glucose-induced expression of the genes for ACC, fatty acid synthase (FASN), and other enzymes by inhibiting transcription factors.11) Moreover, the AMPK pathway is linked to tumor growth and proliferation through regulation of the mammalian target of the rapamycin (mTOR) pathway, which is involved in protein synthesis. Increasing knowledge concerning the mTOR pathway led to a fairly logical set of predictions about the use of rapamycin and its analogues as anti-cancer agents in many experimental models, and rapamycin is now used clinically in the treatment of several cancer cell carcinomas.10) Although there is currently a high level of interest in understanding signaling through the mTOR pathway, clinical updates have indicated that rapamycin is only effective against a few cancers, particularly mantle cell lymphoma, endometrial cancer, and renal cell carcinoma.12) Thus, AMPK is emerging as an interesting metabolic tumor suppressor and a promising target for cancer prevention and therapy.

In this study, we hypothesize that PA regulates proliferation...
and apoptosis through AMPK activation, and thereby provide a mechanism by which PA treatment reduces the incidence of breast cancer.

MATERIALS AND METHODS

Materials PA was isolated as described previously.\(^{[33]}\) PA was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, U.S.A.) and diluted in culture medium for use. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco/BRL Life Technologies (Grand Island, NY, U.S.A.). Antibodies to Caspase-3, Caspase-9, poly(ADP-ribose) polymerase (PARP), P-AMPK, AMPK, FASN, P-ACC, ACC, P-mTOR, mTOR, P-70kDa ribosomal protein S6 kinase (P-p70S6K), p70S6K, P-eukaryotic initiation factor 4E binding protein 1 (P-4EBP1), 4EBP1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from SantaCruz (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Transduction Lab (Lexington, KY, U.S.A.). Super Signal \(^*\) West Pico Chemiluminescent substrate was purchased from PIERCE (Rockford, IL, U.S.A.). AccuPower \(^*\) CycleScript RT PreMix (dT20) was obtained from Bioneer Inc. (Daejeon, Korea). Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). Z-VAD-fmk, AICAR, compound C and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture MCF7 human breast cancer cells were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). Cells were grown in DMEM media supplemented with 10% (v/v) FBS, penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37°C in a humidified CO₂ (5%)-controlled incubator. Stock cultures were sub-cultured every 2—3 days using trypsin 0.23%/ethylenediaminetetraacetic acid (EDTA) 0.02% solution.

Cell Proliferation by CCK-8 Assay Cells were seeded at 5 × 10⁵ cells/mL in 96-well microplates and allowed to attach for 24h. PA was added to the medium at various concentrations up to 50 µM and different duration. After treatment, cell viability and/or proliferation was assessed by Cell Counting Kit-8 (CCK-8) (Rockford, IL, U.S.A.). Briefly, highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium, monosodium salt], produced an orange colored water-soluble product, formazan. The absorbance at 450 nm using microplate reader. Three replicates were carried out for each experimental condition.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) After vehicle and drug treatment, cells were subjected to total RNA isolation by using RNAesy mini kit. RNA concentrations were determined by measuring absorption at 260 nm in a spectrophotometer. Aliquots of 1 µg of total RNA from each sample were reverse transcribed to cDNA using an AccuPower \(^*\) CycleScript RT PreMix (dT20) according to manufacturer’s instructions. PCR primers used in this study included; p53: 5′-CTG ACT GTA CCA CCA TCC-3′ and 5′-CTC ATT CAG CTC TCG GAA CAT CTC GAA GCG-3′; p21: 5′-GCC ACT GTG ATG CGC TAA T-3′ and 5′-GGC GTT TGG AGT GGT AGA AA-3′; VEGF: 5′-GGA TGT CTA TCA GCG CAG CTA C-3′ and 5′-TCA CCG CCT CGG CTT GTC ACA TC-3′; GAPDH: 5′-TAG ACG GGA AGC TCA CTG GC-3′ and 5′-AGG TCC ACC ACC CTG TTG CT-3′. The PCR products were separated on a 2% agarose gel and detected by ethidium bromide staining.

Western Blotting Analysis Cells were incubated with 10 to 50 µM PA for 24h, and washed twice in cold phosphate buffered saline (PBS). Cells were lysed with lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% EDTA, 1% Triton-X100, 0.5% NP-40, 1% propidium iodide (PI), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)) and placed on ice for 1h with occasional vortexing. Centrifugation followed at 10000 rpm for 10 min and each cell lysates (50 µg) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% skim milk in PBS containing 0.05% Tween-20 for 1h at 25°C, then incubated with primary antibodies, followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated immunoglobulin G (IgG) and visualized with enhanced chemiluminescence.

Fatty Acid Synthesis Activity Inhibition of FASN activity in whole cells was measured by the incorporation of \(^{[3H]}\) acetyl-CoA into fatty acids. PA was exposed to the culture medium for 24h. After washing out dead cells, adhesive cells were collected and re-plated with the same number, then \(^{[3H]}\) acetyl-CoA (2 µCi) was added to each well and incubated for 4h at 37°C. Fatty acids were isolated by an initial hypotonic lysis of cells in 50 mM Tris, pH 7.5, followed by extraction in chloroform–water (1:1) for 30 min at room temperature. Lipid phase containing radiolabeled lipids was evaporated under N₂ and then counted by scintillation counter.

Flow Cytometric Analysis Cells with 1 × 10⁵ cells/mL were suspended in 300 µL PBS, and 700 µL EtOH was added while tapping. Cells were incubated at 4°C for 1h and washed with PBS, and suspended in 250 µL of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 µg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was stained by applying 250 µL of propidium iodide (50 µg/mL) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on the BD FACS Canto \(^{[38]}\) flow cytometer using FACS Canto \(^{[39]}\) System Software for the percentage of apoptotic cells and G₀/G₁, S and G₂/M phases of the cell cycle.

Statistical Analysis All values were expressed as means of triplicate assays ± S.E. for each group. One way analysis of variance (ANOVA) was used to analyze differences among multiple comparisons. *p<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

In previous reports, PA inhibited cell growth at 10 µg/mL in HL-60 cells, and induced apoptosis in cells from chronic myeloid leukemia patients.\(^{[14,15]}\) Pentacyclic triterpenes such as PA have demonstrated cytoxicity in multidrug resistant and sensitive leukemia cell lines.\(^{[23]}\) Because PA induces growth inhibi-
tion and apoptosis in leukemia cells, we investigated the effect of PA in breast cancer cells. In this paper, the cytotoxic effects of PA on cell proliferation and apoptosis were evaluated in MCF7 cells. PA inhibited cell growth (Fig. 1A) and arrested the cell cycle at the sub-G1 phase in a dose-dependent manner (Fig. 1B). In addition, PA elevated the levels of p53 and p21, the apoptosis-related genes, and reduced a survival gene, vascular endothelial growth factor (VEGF) (Fig. 1C). To investigate the biochemical features that lead to apoptosis induced by PA, we examined the effects of PA on activation of caspase-3 and -9, and poly(ADP-ribose) polymerase (PARP). MCF7 cells were treated with PA (10, 25, 50 µM) for 24 h and samples were collected and blotted with antibodies that recognize the inactive form of the caspases. Following treatment with 25 µM reductions of the inactive forms of PARP, and caspases-3 and -9 could already be observed; and after treatment of 50 µM PA, the bands completely disappeared, indicating their full activation (Fig. 1D). After pre-incubation of cells for 1 h with 100 µM Z-VAD-fmk, a pan-caspase inhibitor, 50 µM PA treatment for 24 h eliminated apoptotic cells (Fig. 1E) and PARP cleavage (Fig. 1F), indicating the dependence of PA apoptotic activity on caspase activation. Collectively, apoptotic cell death was increased by both 25 and 50 µM PA treatments.

The development of new treatment strategies for cancer patients remains a challenge. In fact, various elements of proliferation and survival signaling pathways are constitutively overactive or dysregulated in most human malignancies. Breast cancer is one of the most complex but complete models that illustrate many levels of signaling pathway dysregulation. Because such aberrant signaling distinguishes malignant cells from normal, they represent possible strategic targets for therapeutic developments against cancer. One such potential target is AMPK. The understanding of AMPK-targeted therapy has become clinically relevant given the association between a lower incidence of cancer and metformin usage in diabetic patients. Metformin has been widely used > 40 years as an anti-diabetic agent to improve glycemic control by enhancing insulin sensitivity in liver and muscle. In addition, given that obesity, insulin resistance, and hyperinsulinemia may play roles in the onset of malignancies including breast cancer, this further supports the observations that metformin users have a low incidence of cancer. AMPK activation has been shown to play a critical role in regulation of energy metabolism under both physiological and pathological conditions, and involves regulation of downstream pathways relevant to the control of cellular proliferation. Therefore, because identification of new AMPK activators is essential for the development of anti-cancer agents, we investigated the effects of PA on AMPK activation. As shown in Fig. 2A (1—4 lane), PA activated AMPK in a dose-dependent manner. To examine the action of PA on AMPK activation, we used a synthetic AMPK inhibitor (compound C) in MCF7 cells. As expected, the el-
Fig. 2. PA Activates AMPK in MCF7 Breast Cancer Cells
Cells were treated with PA (10, 25, or 50 µM) for 24 h, with 10 µM compound C before being stimulated by PA (50 µM) (A), and PA (50 µM) or/and 1 mM AICAR (B). After treatment, cells were lysed and the levels of phosphorylated AMPK (Thr172) and AMPK were determined by Western blot analysis. (C) After pre-exposure of compound C (10 µM), cells were treated for 24 h with PA (50 µM), and the cell viability was determined by CCK-8 assay as described in Materials and Methods. Data are expressed as the percent relative to control. *p < 0.05 (columns 2 vs. 1; 3 vs. 2).

Fig. 3. PA-Induced AMPK Activation Influences Fatty Acid Synthesis-Involved Proteins, FASN and ACC
(A) After exposure to compound C (10 µM) or AICAR (1 mM), cells were treated with PA (10, 25, or 50 µM) for 24 h. Cells were lysed and levels of FASN, phospho-ACC (Ser79), ACC, and GAPDH were determined by Western blot analysis. (B) After pre-exposure to compound C and AICAR, cells were treated for 24 h with PA (50 µM), then incubated with [3H]acetyl-CoA (2 µCi) for 4 h. Cells were collected, washed, recounted, and then cellular lipids were extracted and quantified relative to vehicle-treated controls. Each data point represents the mean±S.E. for 3 independent experiments. *p < 0.05 (columns 2 vs. 1; 4 vs. 2).
The elevated AMPK activity induced by PA was abrogated (Fig. 2A, lane 4, 5 lane). For confirmation, we treated MCF7 cells with both the AMPK activator (AICAR) and PA. Co-treatment with AICAR and PA increased AMPK even more dramatically (Fig. 2B). In addition, PA-induced cell viability reduction was recovered by compound C, an AMPK inhibitor (Fig. 2C). These results strongly suggest, for the first time, that PA-induced AMPK activation regulates MCF7 cell proliferation.

One mode of action involving AMPK activation through which PA may exert its anti-cancer effect is the regulation of fatty acid synthesis. The de novo synthesis of fatty acids is markedly increased in many cancer cells including breast cancer, as a result of the high expression of fatty acid synthase (FASN), a key enzyme for fatty acid synthesis.21) Phosphorylation and activation of AMPK leads to suppression of FASN gene expression,22) and inactivation of ACC.23) These effects cause a reduction in lipogenesis and synthesis of the ACC product malonyl-CoA, resulting in increased fatty acid oxidation.23) Swinnen et al. provided evidence for an AMPK-related link between energy status, tumor-associated lipogenic metabolism, and the malignant phenotype.23,24) Reduced expression of FASN and ACC by activation of AMPK results in a marked decrease in endogenous lipogenesis, and cancer cells stopped proliferating and lost their invasive and tumorigenic properties in vitro and in vivo.10,23) To explore the mechanism underlying the inhibition of growth by PA, we assessed its effect on key enzymes of fatty acid synthesis, with PA at varying concentrations. Fatty acid synthesis activity was measured by the incorporation of [3H]acetyl-CoA into total cellular lipids. The results showed that PA decreased fatty acid synthesis at 50µM, compared to the vehicle treated control (Fig. 3B). These data suggest that PA-induced AMPK activation inhibits fatty acid synthesis and related proteins.

Experimental studies on the effects of AMPK activator have demonstrated that the activation of AMPK reprograms cellular metabolism and enforces metabolic checkpoints by acting on mTOR for regulating cell growth and metabolism.17) Activation of the AMPK pathway reduces mTOR activation and S6K inactivation, with a general reduction of mRNA translation and protein synthesis.8) We therefore observed whether PA inhibited the AMPK-involved mTOR pathway. Results demonstrated that PA decreased mTOR phosphorylation and prevented the phosphorylation of S6K (Fig. 4A). 4EBP1 is a translational repressor whose action is attenuated when phosphorylated by mTOR. PA also reduced phosphorylated 4EBP1 levels (Fig. 4A). To examine the action of PA on mTOR through AMPK activation, we treated MCF7 cells with both the AMPK regulators (compound C or AICAR) and PA. Co-treatment with compound C and PA recovered phospho-mTOR levels (Fig. 4B). These findings indicate that the mTOR/p70S6K/4EBP1 pathway is specifically involved in PA-induced AMPK activation.

In conclusion, this is the first report of PA exhibiting anti-cancer activity through AMPK activation in MCF7 breast cancer cells. When AMPK was activated by PA, de novo fatty acid synthesis was inhibited by regulation of FASN and ACC. Also protein synthesis was regulated by the mTOR/p70S6K/4EBP1 pathway. Therefore we propose that PA, a novel AMPK activator, has a potential to delay cancer progression and induce apoptosis, properties essential for breast cancer treatment.
cancer therapy.

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