Protoapigenone, a Novel Flavonoid, Induces Apoptosis in Human Prostate Cancer Cells through Activation of p38 Mitogen-Activated Protein Kinase and c-Jun NH$_2$-Terminal Kinase 1/2

Hsueh-Ling Chang, Yang-Chang Wu, Jinu-Huang Su, Yao-Tsung Yeh, and Shyng-Shiou F. Yuan

Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan (H.-L.C., Y.-C.W.); Department of Medical Research and Obstetrics and Gynecology, E-DA Hospital, I-Shou University, Kaohsiung, Taiwan (H.-L.C., S.-S.F.Y.); Department of Obstetrics and Gynecology, Kaohsiung Medical University, Kaohsiung, Taiwan (J.-H.S.); Department of Medical Technology, Fooyin University, Kaohsiung, Taiwan (Y.-T.Y.); and Department of Biological Science and Technology, I-Shou University, Kaohsiung, Taiwan (S.-S.F.Y.)

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ABSTRACT

In this study, we investigated the anticancer effect of protoapigenone on human prostate cancer cells. Protoapigenone inhibited cell growth through arresting cancer cells at S and G$_2$/M phases as well as inducing apoptosis. Blockade of cell cycle by protoapigenone was associated with an increase in the levels of inactivated phospho (p)-Cdc25C (Ser$^{216}$) and a decrease in the levels of activated p-cyclin B1 (Ser$^{147}$), cyclin B1, and cyclin-dependent kinase (Cdk) 2. Protoapigenone triggered apoptosis by increasing the levels of cleaved poly(ADP-ribose) polymerase and caspase-3. In addition, activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun NH$_2$-terminal kinase (JNK)1/2 was a critical mediator in protoapigenone-induced cell death. Inhibition of the expression of p38 MAPK and JNK1/2 by pharmacological inhibitors or specific small interfering RNA reversed the protoapigenone-induced apoptosis through decreasing the level of cleaved caspase-3. In contrast, p38 MAPK, but not JNK1/2, was involved in the protoapigenone-mediated S and G$_2$/M arrest by modulating the levels of Cdk2 and p-Cdc25C (Ser$^{216}$). Moreover, in vivo xenograft study showed that protoapigenone had a significant inhibition of prostate tumor growth without major side effects on the mice we tested. This inhibition was associated with induction of apoptosis and activation of p38 MAPK and JNK1/2 in protoapigenone-treated tumor tissues. In conclusion, our results demonstrated protoapigenone suppressed prostate cancer cell growth through the activation of p38 MAPK and JNK1/2, with the potential to be developed as a chemotherapeutic agent for prostate cancer.

Prostate cancer (PCA) is the most common malignancy and the third leading cause of cancer-related deaths among men in the United States (Jemal et al., 2006). Surgery and radiotherapy are the major curative methods for low-to-moderately differentiated PCA (Kish et al., 2001). Once the cancer spreads beyond the pelvis, there is no effective cure for PCA (Jemal et al., 2006), and treatment relies heavily on chemotherapy to control cancer growth. However, the clinically used chemotherapeutic agents remain highly toxic to normal tissues and prompt scientists continue to search for novel agents, which are more effective and less toxic in PCA treatment (Moss and Petrylak, 2006). The use of plant-derived products has shown great promise in cancer therapy by some preclinical and clinical observations (Cragg and Newman, 2005). Flavonoids are polyphenolic compounds that are ubiquitously present in plants, and this work was supported by Grants NSC96-2628-B-037-002-MY3 (to J.-H.S.) and NSC96-2323-B-037-002 (to Y.-C.W.) from the National Science Council, Taiwan, Republic of China, and Grant NHRI-EX96-9306B1 (to S.-S.F.Y.) from National Health Research Institutes, Taiwan, Republic of China. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.107.135442.

ABBREVIATIONS: PCA, prostate cancer; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun NH$_2$-terminal kinase; ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein kinase kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-yridyl)-1H-imidazole; SP600125, anthrax[1,9-cdf]pyrazol-6(2H)-one-1,9-pyrazoloanthrone; PARP, poly(ADP-ribose) polymerase; Cdk, cyclin-dependent kinase; XTT, sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; siRNA, small interfering RNA; BUN, blood urea nitrogen; Cr, creatinine; FITC, fluorescein isothiocyanate.
they have been shown to possess several biological activities at nontoxic concentrations (Ren et al., 2003). Flavonoids have the potential on cancer chemoprevention and chemotherapy for their variety of anticancer activities, including antiproliferation, cell cycle arrest, and induction of apoptosis (Kandaswami et al., 2005; Li et al., 2007). These biological activities are thought to occur through the regulation of signal transduction pathways such as nuclear factor-xB, activator protein-1, or mitogen-activated protein kinases (MAPKs) (Kong et al., 2001; Sarkar and Li, 2004; Fresco et al., 2006).

MAPKs are proline-directed Ser/Thr protein kinases, and they regulate many cellular processes, including cell proliferation, migration, differentiation, and death (Pearson et al., 2001; Ray et al., 2006). Three subfamilies of MAPKs, including c-Jun NH2-terminal protein kinase (JNK), extracellular signal regulating kinase (ERK), and p38 MAPK, have been identified, and they are activated by their upstream MAPK kinase (MKK) (Widmann et al., 1999; Johnson and Lapadat, 2002). MAPK pathway has been implicated in the response of tumor cells to chemotherapeutic drugs, such as paclitaxel (Taxol), etoposide, and cisplatin (Bacus et al., 2001; Brantley-Finley et al., 2003; Brozovic et al., 2004), suggesting that the modulation of MAPK pathway may be a promising target for anticancer drug.

*Thelypteris torresiana* (Gaud) is a fern grown in Taiwan that is used as vegetable or folk medicine. Protoapigenone (Fig. 1A), a new flavonoid compound, is isolated from the whole plant of *T. torresiana*, and it has anticancer activity against breast and hepatocellular cancer cells (Lin et al., 2005). In our pilot study, we observed a significant growth inhibition activity of protoapigenone on prostate cancer cells. Prostate cancer is a major malignancy in men of Western world, and its incidence is growing rapidly in this country. Therefore, in this study, we determined the biological activity and the underlying mechanisms of protoapigenone on prostate cancer cells by using in vitro and in vivo experimental models.

**Materials and Methods**

**Chemicals and Reagents.** Protoapigenone was isolated from the whole plant *Thelypteris torresiana* (Gaud) as described previously.

**Fig. 1.** Effect of protoapigenone on inhibiting the growth of LNCap cells. A, chemical structure of protoapigenone (Lin et al., 2005). B, cells were treated with 2.5, 5, and 10 μM protoapigenone for 12, 24, or 48 h. The cell proliferation rates were determined by XTT assay, and they are presented as mean ± S.D. *, *P < 0.01; **, *P < 0.001. C, morphological changes of cells was photographed under an inverted microscope after treatment with indicated concentrations of protoapigenone (2.5, 5, and 10 μM) for 12 or 24 h.

**Fig. 2.** Induction of apoptosis by protoapigenone in LNCap cells. A, cells were treated with 10 μM protoapigenone for 3 h, and then they were stained with annexin V-FITC (e and f) and DAPI (c and d). B and C, LNCap cells were treated with 2.5, 5, and 10 μM protoapigenone for 12 or 24 h, and then they were analyzed by TUNEL assay (B) and immunoblotting (C). The ratio of annexin V-FITC (A) and TUNEL-staining (B) cells was calculated, and it is presented as mean ± S.D. *, *P < 0.01; **, *P < 0.001.
cell cycle distribution, cells were plated on 6-cm culture dishes, and then they were grown for 24 h before treatment with 2.5, 5, and 10 μM protoapigenone. After treatment for 12, 24, or 48 h, the cytotoxicity of protoapigenone was determined by using XTT cell proliferation assay (Sigma-Aldrich). The concentration of 50% cellular cytotoxicity (IC₅₀) of protoapigenone was calculated, as described previously (Chang et al., 2006).

**Cellular Morphology Analysis.** Cells were grown on the 6-cm culture dishes, and then they were treated with indicated concentrations of protoapigenone (2.5, 5, and 10 μM) for 12 or 24 h. A Nikon TC100 microscope (Ramsey, MN) was used to study the morphological change of protoapigenone-treated cells, as described previously (Yuan et al., 2006).

**Cell Cycle Analysis.** To determine the effect of protoapigenone on cell cycle distribution, cells were plated on 6-cm culture dishes, and then they were treated with 2.5, 5, and 10 μM protoapigenone for 6 or 12 h. After treatment, the cells were harvested by trypsin, fixed with 75% ethanol, washed twice with PBS, and resuspended in the propidium iodide/RNase A (Sigma-Aldrich) solution for 30 min. The DNA content was detected by a FACScan flow cytometry (BD Biosciences, San Jose, CA), and data analysis was done with CellQuest software (BD Biosciences).

**Annexin V Apoptosis Assay.** After treatment with 10 μM protoapigenone for 3 h, cells were washed twice with ice-cold PBS, and then they were stained with binding buffer containing fluorescein-labeled annexin V (1 μg/ml annexin V-FITC; Strong Biotech, Taipei, Taiwan) and 4,6-diamidino-2-phenylindole (Sigma-Aldrich) at 25°C for 15 min. The stained cells were incubated in DAB solution for 15 min in dark. The stained cells were observed with an optical microscope (Axiovert100M; Carl Zeiss). The percentages of annexin V-positive cells were counted in 1000 cells, and the apoptosis index at the early stage was calculated.

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay.** Cells were treated with indicated concentrations of protoapigenone (2.5, 5, and 10 μM) for 12 or 24 h, and then they were stained with TUNEL using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI). In brief, cells were fixed in 4% paraformaldehyde for 30 min after protoapigenone treatment. The fixed cells were incubated with digoxigenin-conjugated dUTP and nucleotide mixture in a recombiant terminal deoxynucleotidyl transferase-catalyzed reaction in a humidified atmosphere for 60 min at 37°C, and then they were immersed in stop buffer for 15 min at room temperature. Cells were washed with PBS, and then they were incubated in DAB solution for 15 min in dark. The stained cells were observed with an optical microscope (Axiovert100M; Carl Zeiss). The percentages of TUNEL-positive cells were counted in 1000 cells, and the apoptosis index in the later period was calculated.

**Immunoblotting Analysis.** The detailed procedures for immunoblotting analysis were followed according to Chang et al. (2006). LNCap cells were treated, harvested, and lysed. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with specific primary antibodies and appropriate secondary antibodies, followed by visualization using the enhanced chemiluminescence kit (Amersham, Chalfont St. Giles, UK).

**siRNA Knockdown.** Knockdown of p38 MAPK and JNK1/2 expression in LNCap cells was achieved by specific siRNAs (Santa Cruz Biotechnology, Inc.). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48-h incubation, the cells were treated with or without protoapigenone, and then they were harvested for instructed analyses.
In Vivo Tumor Xenograft Study. Six-week-old male nude mice (Foxnlnu/Foxnlnu) received s.c. injections with $1 \times 10^6$ LNCap cells at one site of the right flank. When tumors reached an average diameter of 3 mm, the mice were randomly grouped and treated i.p. with protoapigenone or vehicle (PBS) every other day. The high-dose group and low-dose group were given 3.7 $\mu$g protoapigenone (a dose equivalent to the IC$_{50}$ for LNCap cells) and 0.37 $\mu$g protoapigenone (a dose equivalent to 1/10 of the IC$_{50}$ value for LNCap cells). Tumor size and body weight were measured twice a week, and the tumor volumes were calculated according to a standard formula: width$^2 \times$ length/2. After treatment for 5 weeks, the mice were sacrificed by deep anesthesia, and blood sampling by cardiac aspiration was done immediately after anesthesia. Complete blood counts were determined by Sysmex XE-2100 analyzer (TOA Medical Electronics, Kobe, Japan), and the plasma blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase, and alanine aminotransferase levels were determined by Beckman LX20 analyzer (Beckman Coulter, Fullerton, CA).

Immunohistochemistry. Immunohistochemical protocol was followed according to Yeh et al. (2006). In brief, the tissues were fixed overnight in 4% paraformaldehyde, and then dehydrated and coated with wax. Tissue sections were sliced to 4 $\mu$m in thickness, and then they were either dyed with hematoxylin and eosin or immunostained with the primary antibody (mouse monoclonal anti-p-p38, anti-JNK1/2).
p-JNK, and anti-cleaved PARP), followed by Universal LAB + kit/horseradish peroxidase (Dako Denmark A/S, Glostrup, Denmark), or they were counterstained with hematoxylin. The results were captured by the Nikon TS100 microscope, and then they were processed by Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Results

Protoapigenone Inhibited Cell Proliferation in LNCap Cells. We first investigated the antiproliferative effect of protoapigenone on the human prostate cancer cell line LNCap. As shown in Fig. 1B, protoapigenone inhibited cell growth of LNCap cells in a dose- and time-dependent manner. The maximal inhibitory effect was observed after protoapigenone treatment for 48 h, and the IC50 value was 3.7 ± 0.2 μM. Morphological changes in LNCap cells were analyzed at 12 and 24 h after treatment, and protoapigenone-treated cells exhibited cellular shrinkage (Fig. 1C).

Protoapigenone Induced Apoptotic Death in LNCap Cells. We next evaluated the effect of protoapigenone on the induction of apoptosis by annexin V-FITC (labeling phosphatidylserine) and TUNEL assay (detecting DNA fragmentation). After treatment with 10 μM protoapigenone for 3 h, the percentage of annexin V-FITC-positive cells was increased to 24.2 ± 1.3%, compared with 2.7 ± 1.0% in control (Fig. 2A). In addition, protoapigenone treatment increased the percentage of TUNEL-positive cells in a dose- and time-dependent manner (Fig. 2B). Moreover, immunoblotting results confirmed this apoptotic induction, and the levels of cleaved caspase-3 and PARP were increased upon protoapigenone treatment (Fig. 2C).

Protoapigenone Induced Cell Cycle Arrest at S and G2/M Phases. To examine the underlying mechanism responsible for the growth-inhibitory effect of protoapigenone, cell cycle distribution and related regulatory factors were assessed. As shown in Fig. 3A, protoapigenone accumulated cells at S and G2/M phases and thus caused a significant inhibition of cell cycle progression in LNCap cells (Fig. 3A).

Protoapigenone Induced the Activation of p38 MAPK and JNK1/2. The anticancer activity of several flavonoids, including (-)-epigallocatechin gallate and apigenin, are associated with the modulation of MAPK pathway (Van Dross et al., 2003; Kim et al., 2005). Thus, we determined the status of MAPK pathway-related proteins after protoapigenone treatment. Exposure of LNCap cells to protoapigenone for 1 h re-
sulted in a dramatic increase in the phosphorylation of p38 MAPK and JNK1/2 (Fig. 4A). The phosphorylation of MKK3/6 and MKK4, the upstream kinases of p38 MAPK and JNK1/2, was also increased (Fig. 4B). Alternatively, expression of the total forms of MKK3/6, MKK4, p38 MAPK, and JNK1/2 was not altered by protoapigenone treatment (Fig. 4), neither was ERK phosphorylation (data not shown).

To evaluate the role of p38 MAPK and JNK1/2 activation in protoapigenone-induced cell death, LNCap cells were pre-treated for 1 h with pharmacologic inhibitors of p38 MAPK (SB203580) and JNK1/2 (SP600125). The protoapigenone-induced phosphorylation (activation) of p38 MAPK and JNK1/2 was blocked in the presence of SB203580 and SP600125, respectively. (Fig. 5A). Furthermore, inactivation of p38 MAPK and JNK1/2 reversed the protoapigenone-inhibited cell proliferation of LNCap cells (Fig. 5B).

Differential Roles of p38 MAPK and JNK1/2 in Protoapigenone-Mediated Apoptosis and Cell Cycle Arrest. We investigated whether the activation of p38 MAPK and JNK1/2 is involved in protoapigenone-induced apoptosis and cell cycle arrest. As shown in Fig. 6A (left), the protoapigenone-increased apoptotic cells were reversed by SB203580 and SP600125, which blocked the activation of p38 MAPK and JNK1/2, respectively. In addition, the levels of cleaved caspase-3 were abrogated in the presence of SB203580 and SP600125 (Fig. 6A, right). It is interesting to note that selective inhibition of p38 MAPK, but not JNK1/2, significantly attenuated protoapigenone-induced S and G2/M arrest (Fig. 6B, left). The protoapigenone-mediated increase of inactive p-Cdc25C (Ser216) and decrease of Cdk2 were blocked in the presence of SB203580 (Fig. 6B, right). Despite the ability to reverse protoapigenone-induced apoptosis, the inhibition of JNK1/2 had no significant effect on protoapigenone-induced cell cycle arrest (Fig. 6B).

To further analyze the role of p38 MAPK and JNK1/2 in protoapigenone-induced cell death, the siRNAs specific for p38 MAPK and JNK1/2 were applied to knockdown the expression of p38 MAPK and JNK1/2, respectively (Fig. 7A). Compared with oligonucleotide-transfected control cells, transfection of cells with p38 MAPK or JNK1/2 siRNAs reduced protoapigenone-induced apoptosis and the expression of cleaved caspase-3 (Fig. 7, B and D). Selective inhibition of p38 MAPK, but not JNK1/2, released protoapigenone-induced S and G2/M phase arrest by modulating the levels of Cdk2 and inactive p-Cdc25C (Ser216) (Fig. 7, C and 7D). All these results were consistent with the observation from inhibitors of p38 MAPK and JNK1/2 (Fig. 6).

Protoapigenone Suppressed the Growth of Human Prostate Cancer Xenografts via Activating p38 MAPK and JNK1/2 and Inducing Apoptosis. To determine whether protoapigenone suppressed prostate cancer cell growth in vivo, LNCap cells were injected into right flank of nude mice, and the inhibition of tumor growth by protoapigenone was analyzed. After protoapigenone treatment for 5 weeks, the average tumor size in high-dose (1153.0 ± 218.7 mm³) and low-dose (1876.9 ± 428.6 mm³) was significantly smaller than that in the control group (3409.8 ± 704.8 mm³) (Fig. 8A). No significant impairment of hematopoiesis, liver function, or renal function was observed after i.p. injection of protoapigenone, and the body weight had no significant difference between control and protoapigenone-treated group (Table 1). In addition, the levels of cleaved PARP as well as the phosphorylation of p38 MAPK and JNK1/2 were increased in protoapigenone-treated tumor tissues compared with control (Fig. 8B). The unphosphorylated forms of p38 MAPK and JNK1/2 were not altered by protoapigenone treat-
Discussion

Flavonoid compounds are described to play an important role as chemotherapeutic agents (Li et al., 2007). In the present study, we demonstrated that a novel flavonoid compound, protoapigenone, effectively inhibited the prostate cancer cell growth in vitro and in vivo through activation of p38 MAPK and JNK1/2 pathways.

The stress-activated protein kinases JNK1/2 and p38 MAPK are induced in many cell lines when treated with toxic agents, and their activation is involved in apoptosis (Xia et al., 1995; Boldt et al., 2002). It has been reported that activation of p38 MAPK and JNK1/2 is associated with the morphological change, DNA fragmentation, and caspases activation in response to apoptotic agents (MacFarlane et al., 2000; Deschesnes et al., 2001). Anticancer activity of certain flavonoids, such as naringenin and luteolin, induces apoptosis of cancer cells by activating p38 MAPK and/or JNK1/2 (Lee et al., 2005; Gopalakrishnan et al., 2006). After the treatment of LNCap cells with protoapigenone, we observed that protoapigenone caused the activation of p38 MAPK and JNK1/2 pathways, increased levels of cleaved PARP and caspase-3, and eventually apoptosis of prostate cancer cells (Figs. 1, 2, and 4). Caspase-3 is one of the effector caspases and a key protease responsible for PARP cleavage in apoptosis (Duriez and Shah, 1997). The apoptotic events induced by anticancer agent, such as berberine, have been proven to correlate with the activation of p38 MAPK, JNK1/2, and caspase-3 (Hsu et al., 2007). Blockade of p38 MAPK and JNK1/2 with their pharmacological inhibitors or specific siRNAs are associated with a decrease in apoptosis (Kuo et al., 2007; Lin et al., 2007). In the present study, we showed that inactivation of p38 MAPK and JNK1/2 by pharmacological inhibitors or siRNAs for p38 MAPK and JNK1/2 reversed protoapigenone-induced apoptosis and decreased the levels of protoapigenone-induced cleaved caspase-3 (Figs. 5–7). These results implied that p38 MAPK and JNK1/2 played a critical role in protoapigenone-induced apoptosis. Moreover, we observed an increase in the levels of cleaved PARP as well as the activation of p38 MAPK and JNK1/2 in protoapigenone-treated tumors (Fig. 8), suggesting that protoapigenone-induced suppression of prostate tumor growth was associated with activation of p38 MAPK and JNK1/2, and induction of apoptosis.

The p38 MAPK and JNK1/2 pathways are also involved in the cell cycle arrest induced by various anticancer agents, such as flavonoids (Ujiki et al., 2006). Activated p38 MAPK and JNK1/2 regulate the expression of Chk1/2, Cdk2, Cdc25C, cyclin B1, and Cdc2 proteins and thereby cause G1/S or G2/M arrest (Deguchi et al., 2002; Zhang et al., 2002; Frey and Singletary, 2003). In this study, we found that protoapigenone-induced accumulation of prostate cancer cells at S and G2/M phases was associated with an increase in the

Fig. 8. Protoapigenone suppressed the xenograft tumor growth by increasing p38 MAPK and JNK1/2 activation. Male nude mice bearing LNCap prostate tumors were treated with the control solvent or protoapigenone at low dose and high dose for 5 weeks. A, tumor volumes were measured weekly for each group, and the data are presented as mean ± S.D. B and C, tumor samples were analyzed by immunoblotting (B) and immunohistochemical analysis (C). (C1–C3, control group; L1–L3, low-dose group; H1–H3, high-dose group).
levels of inactive p-Cdc25C (Ser216) and a decrease in the levels of p-cyclin B1 (Ser147) and Cdk2 (Fig. 3). Intriguingly, inactivation of p38 MAPK, but not JNK1/2, by inhibitor or siRNA approaches abolished protoapigenone-induced S and G2/M phases arrest in LNCap cells (Figs. 6 and 7). Further studies are required to understand the detailed mechanism how p38 MAPK regulates protoapigenone-induced cell cycle arrest.

In conclusion, our study demonstrated that protoapigenone significantly inhibited the growth of LNCap human prostate cancer cells both in vitro and in vivo, and it provided the underlying mechanism for the antiproliferation activity of protoapigenone. Protoapigenone suppressed the growth of prostate cancer cells without significant hepatotoxicity, nephrotoxicity, and hematological toxicity, rendering it a promising chemotherapeutic agent for prostate cancer in the future.

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References


TABLE 1

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<th>Body weight (g)</th>
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Note: The data are presented as mean ± S.D.


Address correspondence to: Dr. Shyng-Shiou F. Yuan, Department of Medical Research, E-DA Hospital, I-Shou University, 6, E-DA Rd., Jiau-Shu Tsuen, Yan-Chau Shiang, Kaohsiung County, Taiwan 824, Republic of China. E-mail: yuanssf@ms33.hinet.net