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ORIGINAL RESEARCH

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Platycodin D induces apoptosis in human prostate carcinoma cells via ROS-dependent inactivation of the PI3K/AKT/mTOR signaling pathway

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Abstract

Background: Platycodin D is a triterpene saponin present in Platycodi Radix, an herbal medicine used for treating various diseases. Recently, this saponin has been identified as a bioactive natural compound with health benefits, including anticancer properties and no side effects. However, studies on its anticancer effects in human prostate cancer (PCa) cells remain limited. In this study, we elucidated the mechanism of anticancer activity of platycodin D in human PCa cells. **Methods**: After measuring the cytotoxicity of platycodin D, the anticancer potential of this compound was determined by analyzing the induction of apoptotic cell death, DNA damage, reactive oxygen species (ROS) generation, glutathione content, caspase activity and mitochondrial membrane potential (MMP). Additionally, the underlying mechanism of platycodin D-induced apoptosis was determined through various analyses including protein expression analysis. **Results**: Platycodin D treatment induced apoptosis in PCa cells by activating the caspase cascade and reducing the protein expression levels of the inhibitor of the apoptosis family. In addition, platycodin D disrupted mitochondrial integrity by altering Bcl-2 family protein expression and MMP levels, leading to increased cytochrome *c* release into the cytosol. Moreover, platycodin D suppressed the pro-survival phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) (PAM) pathway. Pretreatment with a PI3K inhibitor significantly enhanced the cytotoxic effect of platycodin D on PC-3 cells. Furthermore, platycodin D promoted the generation of ROS, while ROS scavengers restored reduced cell viability by attenuating DNA damage and apoptosis through inhibition of platycodin D-mediated inactivation of the PAM pathway. Platycodin D induces apoptosis in PC-3 cells via ROS-dependent inactivation of the PAM pathway, suggesting that ROS generation plays a key role as an early mediator of platycodin D-mediated anticancer effects. **Conclusions**: Our findings indicate that platycodin D may serve as a potential candidate for inhibiting human PCa cell proliferation.

Keywords

Platycodin D; Apoptosis; Prostate cancer cells; ROS; PI3K/AKT/mTOR

1. Introduction

Traditional medicine has long been used for therapeutic purposes and serves as a valuable resource for discovering new bioactive natural compounds. Over the past several decades, research on natural product-based drugs has made significant contributions to controlling numerous diseases, including cancer [1, 2]. Among these, saponins, a group of secondary metabolites comprising hydrophilic glycosides of steroids and triterpenes, are found across various organisms [3]. *Platycodon grandiflorum* (Jacq.), an herbaceous perennial from the Campanulaceae family, has been widely used as a medicinal herb owing to its diverse health benefits, and platycodin D is a triterpene saponin found in the root of this plant (Platycodi Radix) [4, 5]. Platycodin D has been shown in numerous studies to have a variety of pharmacological effects, including anti-inflammatory, antioxidant, anti-allergy, anti-fibrotic, anti-metabolic syndrome, immunostimulatory, hepatoprotective, neuroprotective and cardiovascular protective effects, with no side effects [6–8].

Meanwhile, the anticancer effects of platycodin D have been extensively documented in recent years across various cancer cell lines and *in vivo* models. These anticancer activities of platycodin D include inhibition of cell cycle progression, angiogenesis, cell invasion and metastasis, while enhancing chemosensitivity to anticancer drugs through multiple mechanisms [9–12]. Furthermore, platycodin D promotes cancer cell apoptosis by regulating

intracellular signaling molecules. For example, Feng *et al.* [13] recently reported that platycodin D exhibits anti-lung cancer activity by inducing apoptosis through inhibition of the transforming growth factor β signaling. Platycodin D induces cytoprotective autophagy-mediated cell death in colon cancer cells, accompanied by changes in the activity of the mitogen-activated protein kinase pathway [14]. In addition, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) (PAM) antiapoptotic signaling, which promotes cell survival and proliferation and contributes to chemoresistance, was inactivated in various cancer cells treated with platycodin D, including endometrial cancer, bladder cancer, acute myeloid leukemia, colorectal cancer, lung cancer and glioma cells [15–18]. However, in a type 2 diabetes mellitus (T2DM) model of cognitive dysfunction, platycodin D alleviated neuronal damage by inhibiting oxidative stress and inducing apoptosis, with AKT phosphorylation playing a central role [19]. Furthermore, activation of the PAM pathway by platycodin D has been shown to attenuate endotoxin-induced inflammation in macrophages [20] and inhibit cisplatin-induced damage in human embryonic kidney cells while reducing reactive oxygen species (ROS) levels [21]. These studies indicate that the antioxidant activity of platycodin D acts as a defense against normal cell damage from various stimuli [22–24]. Conversely, oxidative stress is known to be a critical factor in the initiation of apoptosis in cancer cells [25, 26], and inactivation of the PAM pathway by oxidative stress may be a potential therapeutic target for the anticancer effects of platycodin D [17, 21].

Abnormal PAM signaling is implicated in various mechanisms contributing to cancer development, either directly, such as through the regulation of mitochondrial bioenergetics, or indirectly, via a metabolic byproduct [27, 28]. Platycodin D has been reported to arrest growth and induce apoptosis in human prostate cancer (PCa) cells [29] and enhance the anticancer activity of the anticancer sorafenib [30], but the role of the PAM signaling in its anticancer activity remains unclear. Furthermore, since activation of the PAM signaling and maintenance of redox homeostasis are mechanisms that confer a selective advantage to cancer cells, inhibition of ROS in platycodin D-induced apoptosis may be responsible for the inactivation of this pathway. Therefore, this study aimed to elucidate the role of the ROS-mediated PAM signaling in the anticancer activity of platycodin D in human PCa cells.

2. Materials and methods

2.1 Chemicals and reagents

Platycodin D (SMB00424), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1105546-02-8), necrostatin-1 (480065), carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk) (C2105), paraformaldehyde (30525-89-4), and an enhanced chemiluminescence (ECL) kit (WBAVDCH01) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All materials required for cell culture were purchased from WelGENE (Gyeongsan, Republic of Korea). Dimethyl sulfoxide (DMSO) (67-68-5), N-acetyl-L-cysteine (NAC) (616-91-1), 4′,6′-diamidino-2-phenylindole (DAPI) (D21490), glutathione (GSH) fluorescence detection kit (15840903), 2′,7′-dichlorofluorescein diacetate (DCF-DA) (2500553), and MitoTracker^{TM} Red (M22425) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (556570) and LY294002 (#9901) were purchased from BD Biosciences (Franklin Lakes, NJ, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA). Immun-Blot® membranes (#1620177) and mitochondrial/cytosolic protein fractionation kits (40015) were manufactured by Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and Active Motif, Inc. (Carlsbad, CA, USA). Antibodies against the target proteins were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Abcam Ltd. (Cambridge, UK) and Cell Signaling Technology, Inc. Caspase activity assay kits (Caspase-3 activity assay kit, ab3940; Caspase-8 activity assay kit, ab39700; Caspase-9 activity assay kit, ab65615), 8-hydroxy-2′-deoxyguanosine (8-OHdG) enzyme-linked immunosorbent assay (ELISA) kit (ab201734), and 5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) (ab288313) were purchased from Abcam Ltd. The comet assay kit (4250-050-K) used in this study was manufactured by Trevigen, Inc. (Gaithersburg, MD, USA).

2.2 Cell culture and platycodin D treatment

Human PCa cell lines (PC-3 and DU145 cells; CRL-1435™ and HTB-81D™) procured from the American Type Culture Collection (Manassas, VA, USA), were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Platycodin D was prepared as a stock solution by dissolving it in DMSO and then diluted to the appropriate concentration in culture medium prior to treating cells.

2.3 Cell viability assay

To treat with platycodin D, cells were stabilized for 24 h, and then various concentrations of platycodin D were added to the medium and cultured for 48 h. For pretreatment conditions, cells were incubated with or without 20 μM z-VAD-fmk, 50 μM necrostatin-1, 10 mM NAC, or 10 μM LY294002 for 1 h, followed by treatment with or without 30 μM platycodin D for 48 h. Cell viability was assessed using an MTT assay following a previously described method [31]. Cells were imaged with an optical microscope (Zeiss Axioscope 5, Carl Zeiss, Oberkochen, BW, Germany).

2.4 Assessment of apoptosis by nuclear morphology change

To examine the effects of platycodin D on nuclear morphology, DAPI staining was performed. Briefly, cells cultured for 48 h in the absence or presence of platycodin D were collected, washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and stained with 2.5 μM DAPI solution at room temperature (RT) [32]. The morphology of DAPI-stained nuclei was observed using a fluorescence microscope (ZEISS

Axio Scope A1 Carl Zeiss).

2.5 Quantitative assessment of apoptosis using flow cytometry

The degree of apoptosis induction was examined using an annexin V-fluorescein isothiocyanate (V-FITC) apoptosis detection kit. Briefly, cells treated with platycodin D were suspended in a binding buffer and reacted with annexin V-FITC and propidium iodide (PI) buffer for 20 min at RT following a previously described method [32]. The cell suspension was analyzed with a flow cytometer (Guava EasyCyte 8HT Flow Cytometer, Millipore Corporation, Hayward, CA, USA).

2.6 Protein extraction and immunoblotting

Whole cell lysates from cells cultured under various treatment conditions were prepared from cells according to previous methods [33]. Mitochondrial and cytoplasmic proteins were isolated using a mitochondrial and cytosolic protein fractionation kit. Equal amounts of proteins were fractionated by gel electrophoresis and immediately transferred onto Immun-Blot membranes. The membranes were hybridized with primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were then visualized using an ECL kit. β-actin and cytochrome *c* oxidase subunit IV (COX IV) were probed as loading controls for cytosolic and mitochondrial proteins, respectively.

2.7 Caspase activity assay

The activities of caspase-3, -8 and -9 were measured using caspase activity assay kits, which detect the hydrolysis of fluorescent substrate peptides by activated caspases. After treatment with platycodin D, the supernatant was collected from each well, and an equal amount (50 μL) of caspase substrate diluted 1:200 in assay buffer was added and reacted at 37 ℃ with 5% CO_2. For each caspase activity, the concentration of p-nitroaniline released from the substrates was then determined using a microplate reader set at a 400-nm excitation filter and 505-nm emission filter [34]. Results are presented as fold increase in fluorescence compared to untreated cells treated with platycodin D in each well, in replicates of at least three independent experiments.

2.8 Mitochondrial membrane potential (MMP) analysis

To analyze MMP, an indicator of mitochondrial membrane stability, JC-1 dye, a cationic carbocyanine dye, was used. JC-1 shows voltage-dependent accumulation in mitochondria and begins to form J aggregates in mitochondria. Since JC-1 remains as a monomer and fluoresces green upon depolarization of the mitochondrial membrane, the strong green fluorescence indicates loss of MMP. After treatment with platycodin D for 48 h, the collected cells were washed with PBS and then stained with 10 μM JC-1 for 30 min. The frequency of JC-1 aggregates and monomers was immediately monitored using flow cytometry, as previously described [35].

2.9 ROS production analysis

ROS levels were measured by staining with DCF-DA, which fluoresces upon oxidation by ROS, following the manufacturer's instructions. Briefly, platycodin D-treated cells with or without 10 mM NAC were incubated with 2.5 μM DCF-DA solution, and intracellular ROS levels were measured using flow cytometry. The levels of DCF-DA fluorescence in the cells were also detected by fluorescence microscopy [36]. Additionally, cells were stained with the mitochondrial marker MitoTracker Red. Nuclei were counterstained with DAPI and images were obtained using a fluorescence microscope [36].

2.10 Calculation of GSH/oxidized glutathione (GSSG) ratio

The antioxidant activity of platycodin D was assayed using a GSH assay kit that determines reduced (GSH) and oxidized (GSSG) glutathione levels. Briefly, cell pellets were washed with PBS, suspended in 5% salicylic acid, centrifuged, and the supernatant was separated for measurement of total glutathione (GSH and GSSG) and GSSG. Standard and sample dilutions and assays were performed according to the experimental method provided in the kit, and absorbance was measured at 405 nm using a microplate reader. The relative GSH ratio was analyzed by subtracting the GSSG concentration from the total GSH.

2.11 Comet assay

To assess DNA damage, cell suspensions treated with platycodin D with or without NAC were mixed with molten low-melting point agarose according to the instructions of the comet assay kit. The cell-agarose mixture was spread on slides preheated to 37 °C and left at 4 ℃ for 20 min to solidify the agarose. The slides were immersed in the dissolution solution at 4 ℃ for 1 h, and then left in the alkaline dissolving solution at RT for 20 min. The slides were electrophoresed in alkaline electrophoresis buffer at 4 ℃ for 30 min at 1 V/cm, washed with ultrapure water, and dehydrated in ethanol for 10 min. The cells attached to the slides were stained with the dye (SYBR Gold) provided in the kit, washed with ultrapure water, dried, and then images were obtained under a fluorescence microscope.

2.12 Detection of 8-OHdG levels

To assess the degree of oxidative DNA damage, an ELISA kit was used to measure the level of intracellular 8-OHdG, an oxidized derivative of guanosine. According to the manufacturer's protocol, DNA isolated from cells was reacted with a DNA digestion mixture provided in the kit. The cells were then mixed with a buffer and standards and reacted with an 8-OHdG antibody. The cells were washed with a buffer, and the absorbance was analyzed at 405 nm using an ELISA reader (MicroScan autoSCAN-4 System, Beckman, Brea, CA, USA), as previously reported [37].

2.13 Statistical analysis

All results were analyzed using GraphPad Prism 5.03 software (GraphPad Software Inc., La Jolla, CA, USA) using an unpaired two-tailed Student's *t*-test and one-way analysis of variance. All data are presented as the mean ± standard deviation (SD) and all experiments were performed independently at least 3 times, with *p* values less than 0.05 considered significant.

3. Results

3.1 Platycodin D decreased cell survival and induced apoptosis in PCa cells

As shown by the MTT assay results in Fig. 1A,B, platycodin D decreased the cell viability of PC-3 and DU145 cells in a dose-dependent manner. Fig. 1C shows the morphological differences between control cells and those treated with 30 μM platycodin D. Compared with the control cells, platycodin D-treated cells exhibited severe morphological distortion, branching and loss of contact with neighboring cells. In addition, DAPI staining showed chromosomal fragmentation and condensation in platycodin D-treated cells, hallmarks of apoptosis in the nucleus (Fig. 1D,E). Flow cytometry was performed to determine the frequency of cell death induced by platycodin D treatment. As shown in Fig. 1F, as the concentration of platycodin D treatment increased, the proportion of annexin-positive (apoptotic) cells gradually increased compared to the untreated control cells, indicating that platycodin D inhibited PC-3 and DU145 cell proliferation by inducing apoptosis. Since PC3 cells showed slightly higher cytotoxicity to platycodin D than DU145 cells, subsequent experiments were performed using PC-3 cells.

3.2 Platycodin D-induced caspase-dependent apoptosis in PC-3 cells

To explore the underlying mechanism of apoptosis induced by platycodin D in PC-3 cells, we analyzed changes in several apoptosis-regulating proteins. As shown in Fig. 2A, platycodin D exposure reduced the levels of the inactive forms of caspases (caspase-8, -9 and -3), whereas it increased poly (adenosine diphosphate-ribose) polymerase (PARP) degradation and the activity of each caspase (Fig. 2B). In addition, platycodin D suppressed the expression of inhibitor of apoptosis protein (IAP) family proteins, including cellular inhibitor of apoptosis protein (cIAP)-1 and cIAP2 (Fig. 2A). Moreover, platycodin D promoted the expression of Bax, a representative apoptosis-promoting protein, but reduced the expression of Bcl-2, a key anti-apoptosis protein (Fig. 2C). Next, to determine whether cell death by platycodin D was caspase-dependent, a cell-permeable pan-caspase inhibitor, z-VAD-fmk, was used. As shown in Fig. 2D, z-VAD-fmk significantly rescued cells from platycodin D-induced cytotoxicity. In contrast, necrostatin-1, a selective blocker of necroptosis, failed to abrogate the effects of platycodin D (Fig. 2D), indicating that platycodin D induces caspase-dependent apoptosis rather than necrosis in PC-3 cells.

3.3 Platycodin D increased mitochondrial impairment in PC-3 cells

To explore the role of mitochondria in platycodin D-induced apoptosis, we examined its effect on mitochondrial permeabil-

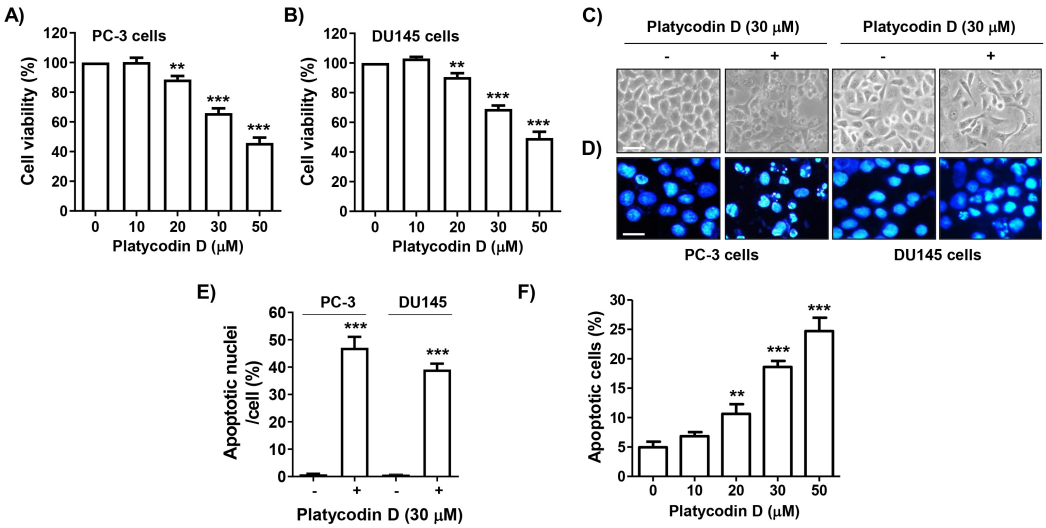

FIGURE 1. Induction of apoptosis by platycodin D in PCa cells. (A,B) Cell viability of PC-3 (A) and DU145 cells (B) treated with various concentrations of platycodin D for 48 h was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (C) After treating cells with 30 μM platycodin D for 48 h, cell morphology was imaged under an optical microscope (magnification: 200×; Scale bar: 100 μm). (D) Morphological changes in 4′,6′-diamidino-2-phenylindole (DAPI)-stained nuclei were captured under a fluorescence microscope (magnification: 400×; Scale bar: 50 μm). (E) The frequencies of apoptotic nuclei are expressed as a percentage of the total number of cells. (F) Cells stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) were analyzed for the degree of induced cell death using flow cytometry, and the percentage of annexin V-positive cells is presented. (A,B,E,F) The results are presented as mean ± SD (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control cells.

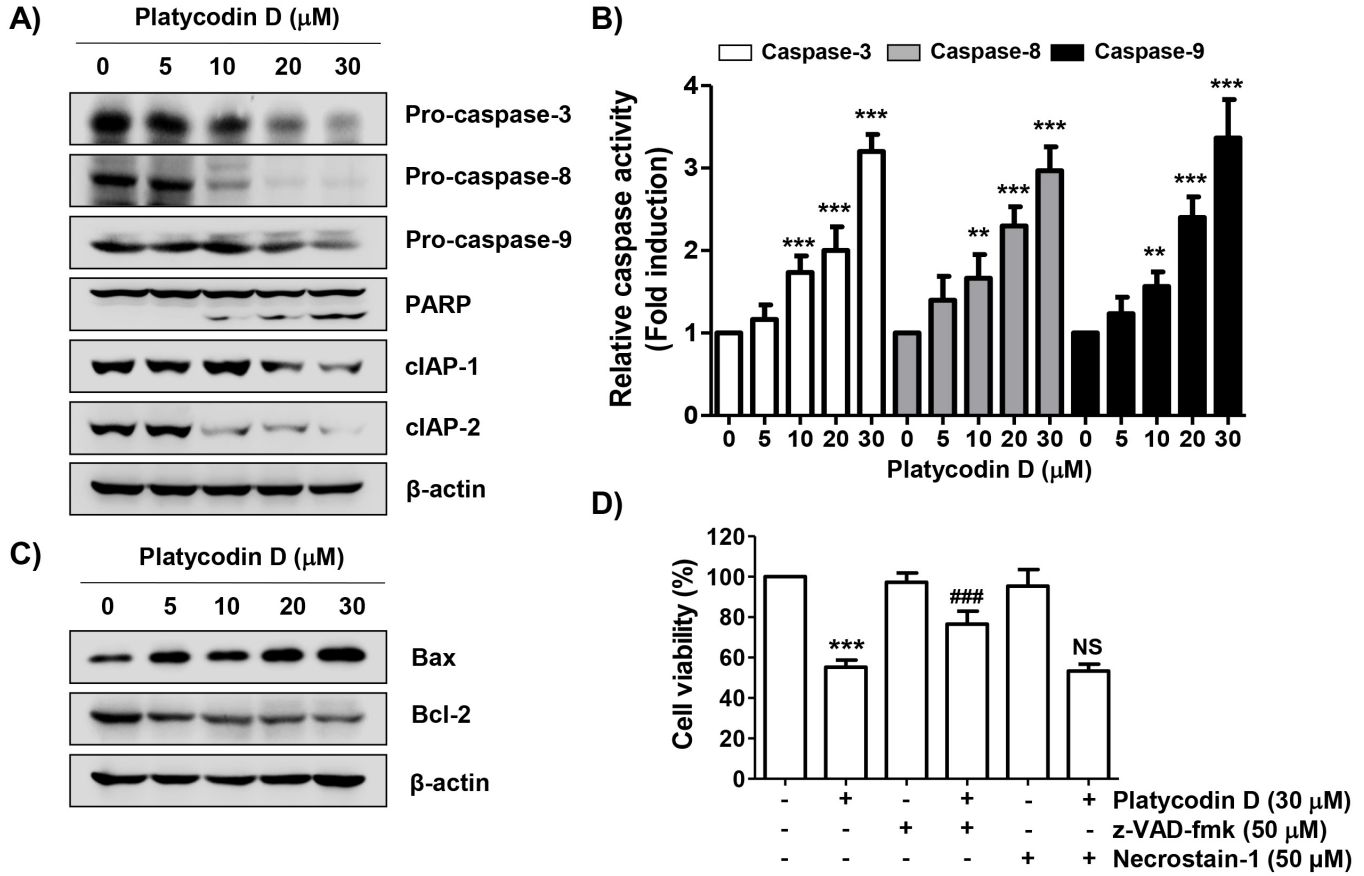

FIGURE 2. Effects of platycodin D on the activation of caspases and the expression of inhibitor of apoptosis proteins and Bcl-2 family proteins in PC-3 cells. (A) After 48 h of treatment with the indicated concentrations of platycodin D, changes in the expression of each protein were evaluated by immunoblotting for total protein. (B) The activity of each caspase was expressed relative to those in the control cells. (C) Bax and Bcl-2 protein expression was examined using immunoblotting. (D) Cells pretreated with 50 μM z-VAD-fmk or 50 μM necrostatin-1 for 1 h were exposed to 30 μM platycodin D for 48 h, and the cell viability was analyzed using the MTT assay. (B and D) The results are presented as mean ± SD (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control cells; $^{\#\#\#}p < 0.001$ versus platycodin D-treated cells; NS, not significant. PARP: poly (adenosine diphosphate-ribose) polymerase; z-VAD-fmk: carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; cIAP: cellular inhibitor of apoptosis protein.

ity. Flow cytometry results showed that platycodin D treatment disrupted MMP, an indicator of mitochondrial stability (Fig. 3A,B). JC-1 staining revealed a shift from J-aggregates to monomers in platycodin D-treated cells, indicating MMP loss. This was associated with a decrease in MitoTracker fluorescence intensity, which, when oxidized in living cells, is sequestered into mitochondria based on membrane potential, confirming a loss in MMP (Fig. 3C). Moreover, platycodin D-induced MMP loss was associated with an upregulation of cytochrome *c* expression in the cytosolic fraction and a concomitant downregulation in the mitochondrial fraction (Fig. 3D), consistent with mitochondrial impairment.

3.4 Platycodin D-induced ROS accumulation in PC-3 cells

To determine whether ROS generation was associated with platycodin D-induced apoptosis, flow cytometry analysis revealed that ROS production increased within 30 min of platycodin D treatment, peaked after 1 h, and then gradually declined (data not shown). However, no significant ROS production was observed when platycodin D alone was treated, and under conditions of pretreatment with NAC, a ROS scavenger, ROS production induced by platycodin D was significantly reduced to control levels (Fig. 4A,B). In line with these results, fluorescence microscopy revealed stronger green fluorescence, indicating ROS accumulation, in platycodin D-treated cells compared to untreated controls, while NAC pretreatment markedly reduced this fluorescence (Fig. 4C). More, of the GSH/GSSG ratio was reduced in cells treated with platycodin D, which was significantly restored in the presence of NAC (Fig. 4D). These results imply that the apoptosis of PC-3 cells induced by platycodin D may be associated with increased oxidative stress.

3.5 Platycodin D triggered ROS-mediated DNA damage in PC-3 cells

We further analyzed whether the induction of cytotoxicity by platycodin D was correlated with DNA damage. Immunoblotting results demonstrated that the expression of phosphorylated H2AX (γH2AX), a DNA damage marker, was

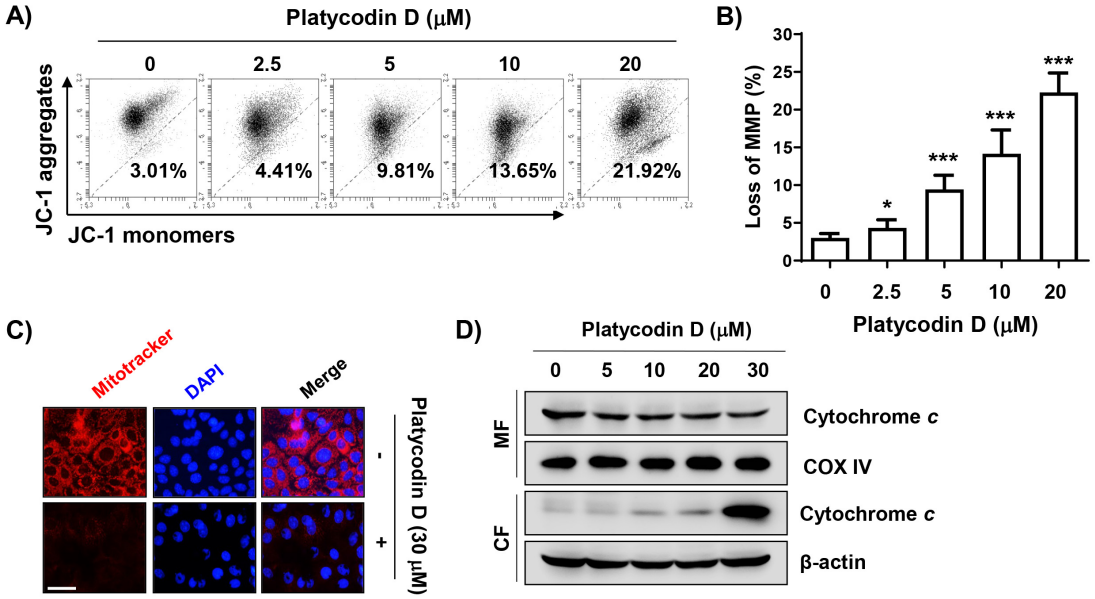

FIGURE 3. Induction of mitochondrial impairment by platycodin D in PC-3 cells. Cells were cultured in medium containing platycodin D at the indicated concentrations for 48 h. (A,B) To measure mitochondrial membrane potential (MMP) changes, cells stained with 5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) were subjected to flow cytometry. (A) Representative histogram profiles with values at the bottom of the box indicating the frequency of cells with depolarized mitochondrial membranes. (B) Statistical analysis of MMP loss after treatment of cells with platycodin D. The results are presented as mean ± SD (n = 3). *$p < 0.05$ and ***$p < 0.001$ versus control cells. (C) Fluorescence microscopy images of MitoTracker Red-1-stained cells. Nuclei were stained with DAPI (blue; Scale bar: 100 μm). (D) After separation of mitochondrial (indicated as MF) and cytoplasmic fractions (indicated as CF), the expression levels of cytochrome *c* were measured using immunoblotting. DAPI: 4′,6′-diamidino-2-phenylindole; COX: cytochrome *c* oxidase.

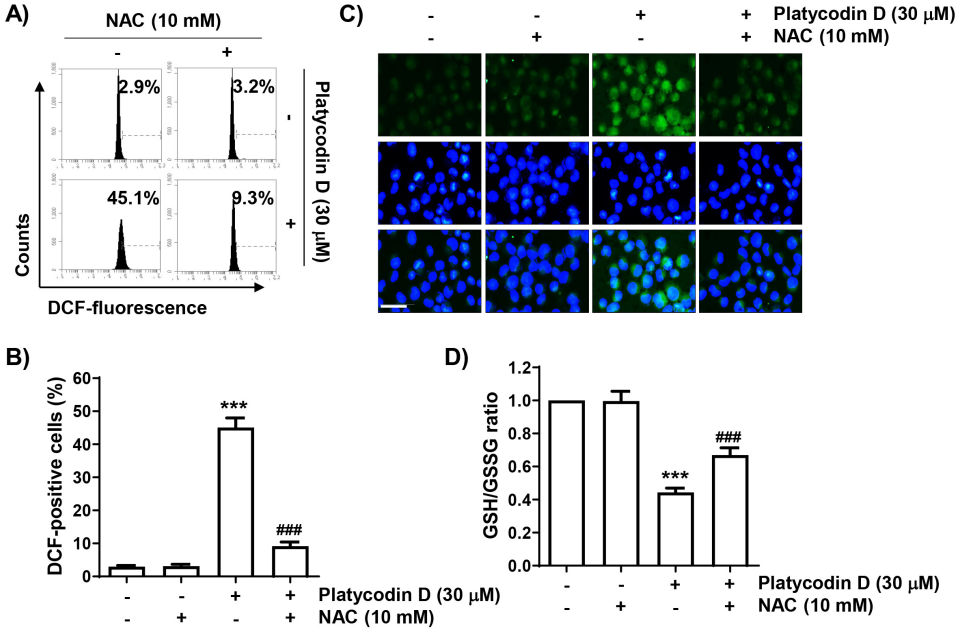

FIGURE 4. Increase in reactive oxygen species (ROS) production and decrease in glutathione (GSH) and oxidized glutathione (GSSG) in platycodin D-treated PC-3 cells. Cells treated with 10 mM N-acetyl-L-cysteine for 1 h were then treated with 30 μM platycodin D for either 1 h (A–C) or 48 h (D). (A,B) Intracellular ROS levels were analyzed by flow cytometry after 2′,7′-dichlorofluorescein diacetate (DCF-DA) staining. (A) Representative DNA histograms are shown. (B) The percentage of DCF-positive cells in each experimental group is presented. (C) ROS generation (green) was confirmed by fluorescence microscopy, with nuclei stained using DAPI (blue; Scale bar: 100 μm). (D) The GSH/GSSG ratio was determined using a GSH assay kit. (A,B,E) The results are presented as mean ± SD (n = 3). ***$p < 0.001$ versus control cells; ###$p < 0.001$ versus platycodin D-treated cells. NAC: N-acetyl-L-cysteine.

increased in platycodin D-treated PC-3 cells (Fig. 5A). This was confirmed by the comet assay showing DNA strand breaks and increased 8-OHdG levels, indicative of oxidative DNA damage (Fig. 5B,C). However, γH2AX expression was almost completely abolished in platycodin D-treated cells pretreated with NAC (Fig. 5A). Similarly, NAC pretreatment reduced DNA-tail-like structures and 8-OHdG levels as a result of blocked ROS production (Fig. 5B,C), suggesting that ROS are essential for platycodin D-induced DNA damage in PC-3 cells.

3.6 Platycodin D-induced apoptosis via ROS-dependent inactivation of the PAM signaling in PC-3 cells

Finally, to investigate whether the PAM signaling plays a key role in the anticancer activity of platycodin D in PC-3 cells, the results of immunoblotting showed that the expression of phosphorylated PI3K (p-PI3K, Tyr458, Tyr199) decreased as the platycodin D concentration increased. Similarly, the phosphorylation of mTOR as well as AKT, downstream targets of PI3K, was significantly suppressed by platycodin D (Fig. 6A), while total protein levels remained largely unchanged. Pretreatment with NAC completely rescued platycodin D-induced dephosphorylation of these proteins and preserved the expression of PARP without degradation (Fig. 6B). Moreover, LY294002, a PI3K inhibitor, significantly enhanced platycodin D-induced apoptosis, increased ROS production, decreased the GSH/GSSG ratio and intensified the platycodin D-mediated reduction in cell survival, whereas NAC markedly attenuated these effects (Fig. 7). These findings demonstrate that platycodin D inhibits cell viability and promotes apoptosis in PC-3 cells by inactivating the ROS-dependent PAM signaling pathway.

4. Discussion

Abnormalities in intracellular signaling pathways, including the PAM signaling pathway, which are common in most cancer cells, increase resistance to apoptosis, a key characteristic acquired by normal cells as they transform into cancer cells [38]. Therefore, identifying therapeutic agents that effectively induce apoptosis through specific signaling pathways in cancer cells is a promising approach for anticancer drug development. Among the primary factors initiating typical apoptosis, caspases play a key role as executioners and are classified into extrinsic and intrinsic pathways based on the initiating caspase [39, 40]. The extrinsic pathway, characterized by death receptor (DR)-mediated activation, is activated when death ligands bind to DRs on the cell membrane surface, leading to the activation of caspase-8, while the mitochondria-mediated intrinsic pathway requires the activation of caspase-9 due to mitochondrial dysfunction. Consequently, caspase-8 and -9 are classified as initiating caspases for their respective

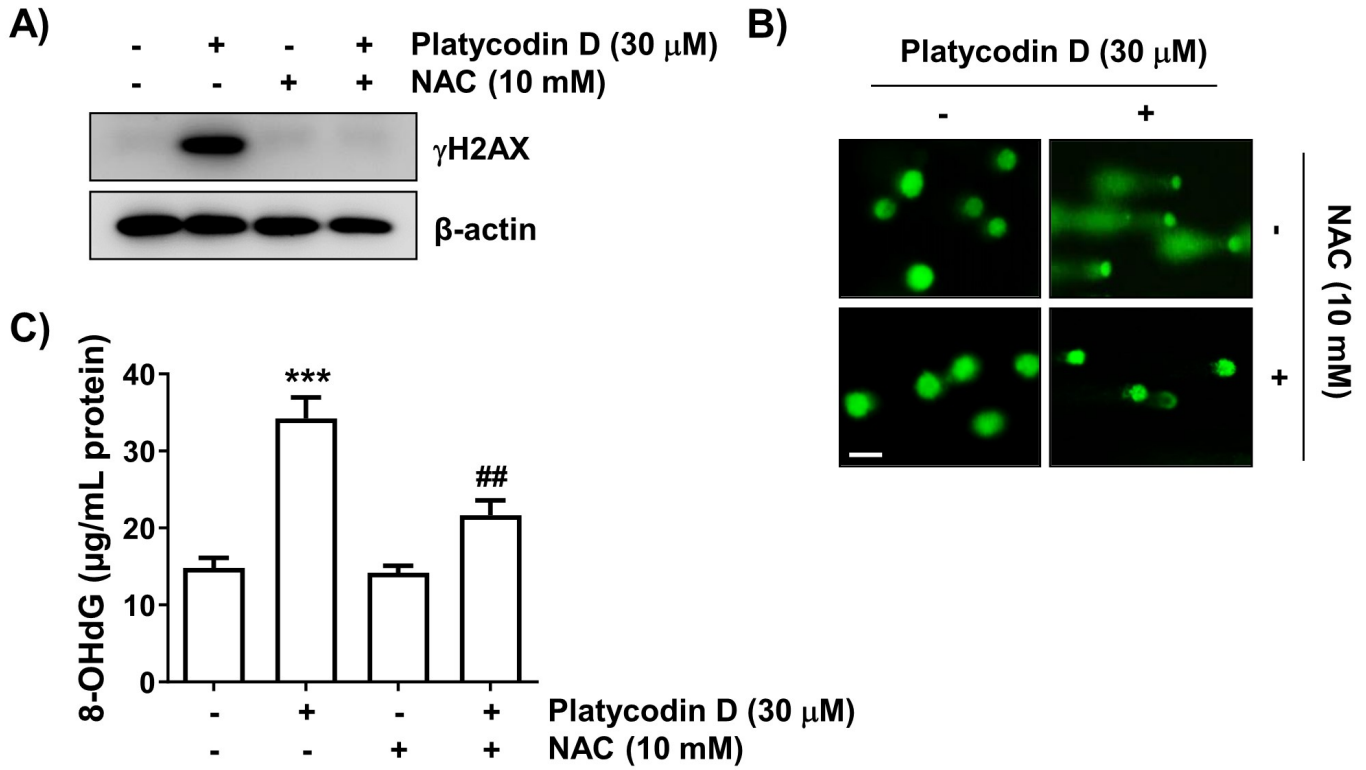

FIGURE 5. Induction of ROS-dependent DNA damage in platycodin D-treated PC-3 cells. Cells were pretreated or not with 10 mM NAC for 1 h and then treated with 30 μM platycodin D for 48 h. (A) Immunoblotting was performed to evaluate changes in the expression of phosphorylated H2AX (γH2AX). (B,C) DNA damage was evaluated by the comet assay (Scale bar: 100 μm) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels. (B) Representative fluorescence images from the comet assay are shown. (C) Nuclear 8-OHdG levels were determined using an enzyme-linked immunosorbent assay kit. The results are presented as mean ± SD (n = 3). ***$p < 0.001$ versus control cells, ##$p < 0.01$ versus platycodin D-treated cells. NAC: N-acetyl-L-cysteine.

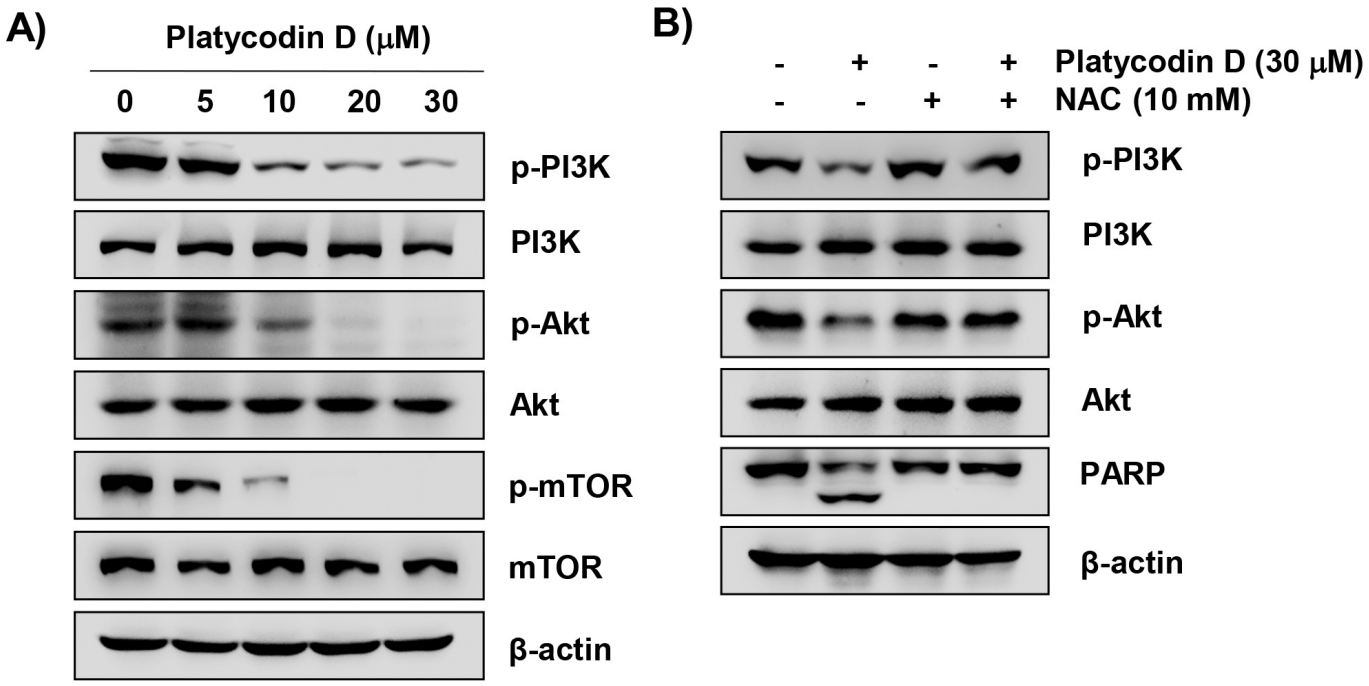

FIGURE 6. ROS-dependent inactivation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) (PAM) signaling by platycodin D treatment in PC-3 cells. Cells were treated with the indicated concentrations of platycodin D for 48 h (A), or pretreated with 10 mM NAC for 1 h or not, then treated with 30 μM platycodin D for 48 h (B), and the isolated total proteins were used for immunoblotting. NAC: N-acetyl-L-cysteine; PARP: poly (ADP-ribose) polymerase.

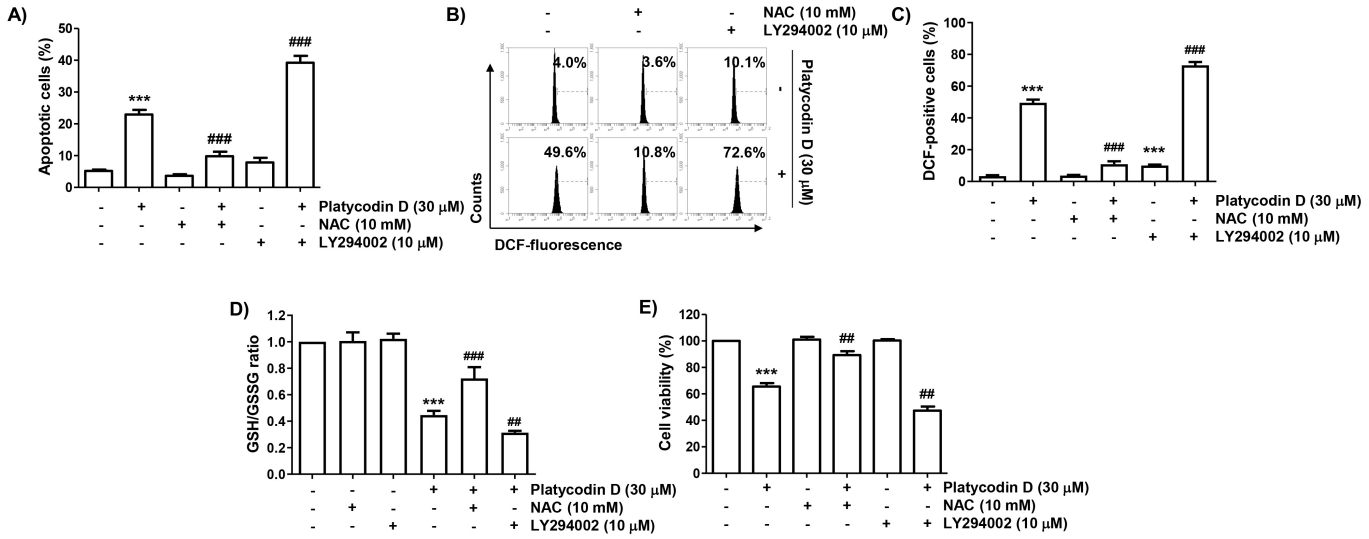

FIGURE 7. Role of PAM signaling and ROS in platycodin D-triggered reduction in cell viability and induction of apoptosis in PC-3 cells. Cells were cultured in medium with or without NAC or LY294002 for 1 h and then treated with platycodin D for an additional 48 h (A,D,E) or 1 h (B,C). (A) Cells stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) were analyzed for the degree of induced cell death using flow cytometry, and the percentage of annexin V-positive cells is presented. (B,C) ROS levels were examined by flow cytometry after DCF-DA staining. Representative histograms (B) and the percentage of DCF-positive cells (C) in each experimental group are shown. (D,E) The GSH/GSSG ratio was calculated using a commercially available kit (D), and cell viability was analyzed using the MTT assay (E). The results are presented as mean ± SD, with n = 3 in each group. ***$p < 0.001$ versus control cells; ##$p < 0.01$ and ###$p < 0.001$ versus platycodin D-treated cells. NAC: N-acetyl-L-cysteine; DCF: 2′,7′-dichlorofluorescein; GSH: glutathione; GSSG: glutathione oxidized glutathione.

pathways, whereas caspase-3 and -7, which are activated by caspase-8 and -9 and promote the completion of apoptosis, are considered effector caspases. However, the activity of caspases can be directly or indirectly regulated by members of the IAP family [40, 41]. In this study, the significant inhibition of cell viability of PCa cells by platycodin D was closely related to the induction of apoptosis (Fig. 1). Mechanistically, the activities of caspase-3, as well as caspase-8 and -9, were statistically significantly upregulated in cells treated with platycodin D, whereas the expression of proteins belonging to the IAP family was downregulated (Fig. 2). Additionally, the degradation of PARP, a key substrate of effector caspases and a DNA repair enzyme [10, 42], was increased with platycodin D treatment. Furthermore, the decrease in viability of platycodin D-treated PC-3 cells was significantly blocked by a pan-caspase inhibitor, though not by a necrosis inhibitor (Fig. 2). Therefore, our findings indicate that the proapoptotic effect of platycodin D in PC-3 cells is achieved via caspase-dependent apoptosis involving intrinsic and extrinsic pathways rather than necrosis.

The Bcl-2 family proteins, which include factors that inhibit or promote apoptosis within the intrinsic pathway, are critical in regulating pore formation in the mitochondrial outer membrane. This process facilitates the cytosolic release of cytochrome *c*, activating the intrinsic caspase cascade pathway and leading to cell destruction [39, 43]. Consistent with previous studies on multiple myeloma, colon cancer and bladder cancer cells [12, 14, 17], platycodin D treatment enhanced the levels of the proapoptotic Bax, which induces apoptosis by disrupting mitochondrial integrity and increasing outer membrane permeability (Fig. 2). In contrast, platycodin D decreased the expression of antiapoptotic Bcl-2 that supports cell survival by suppressing apoptotic activity. Therefore, the loss of MMP, indicating mitochondrial dysfunction in cells treated with platycodin D, may be due to an increase in the Bax/Bcl-2 expression ratio, which would lead to elevated cytosolic cytochrome *c* levels (Fig. 3), highlighting the importance of mitochondria in platycodin D-induced PC-3 cell apoptosis.

Many previous reports have shown that platycodin D blocks oxidative stress-mediated cell injury by inhibiting ROS production in various disease models [21–24]. In addition to the ROS-scavenging ability of platycodin D, it can also block oxidative stress by its ability to modulate the antioxidant GSH. Recently, Song *et al*. [23] reported that platycodin D enhanced GSH activity in an animal model of high glucose-induced diabetic retinopathy, which is consistent with the results in a proximal tubular cell line [44]. However, in human bladder cancer, breast cancer and leukemia cells, platycodin D has been shown to promotes apoptosis through the production of ROS [17, 26]. In these studies, inhibition of oxidative stress by platycodin D in normal cells was associated with recovery from mitochondrial damage, contrary to that in cancer cells. Increasing evidence supports that mitochondrial damage-linked ROS accumulation is crucial for apoptosis; an intracellular redox imbalance leading to excessive ROS production promotes apoptosis and drives DNA oxidation [45, 46]. Furthermore, proapoptotic Bax family members can decrease ROS production in mitochondrial respiration, whereas anti-apoptotic members promote ROS production, thereby promoting cancer cell viability [39, 43]. In platycodin D-treated PC-3 cells, ROS production was significantly increased, while the GSH/GSSG ratio was decreased (Fig. 4). The expression of γH2AX, formation of comet tails, and 8-OHdG levels also markedly increased in platycodin D-treated cells. Furthermore, NAC pretreatment effectively blocked platycodin-induced ROS production and reversed DNA damage (Fig. 5). These findings showed that platycodin D promotes DNA damage and apoptosis, underscoring the functional importance of oxidative damage in platycodin D-induced cytotoxicity in PC-3 cells. However, further confirmation is needed to determine whether the decreased GSH by platycodin D was a major factor in ROS generation.

According to Zhou *et al*. [29], platycodin D induced apoptosis in PCa cells by increasing the activity of forkhead box O3 (FOXO3a), a primary tumor growth inhibitor, without exhibiting toxicity in normal prostate cells. Another study showed that this compound promoted sorafenib-mediated cytotoxicity in PC3 cells; however, this effect was observed only in AKT-positive and phosphatase and tensin homolog (PTEN)-negative cells [30], suggesting that platycodin D may act as an AKT inhibitor or FOXO3a agonist. As a tumor suppressor, PTEN negatively regulates PAM signaling, which is also suppressed by FOXO3a activation, leading to overexpression of cell cycle arrest-inducing factors and ultimately contributing to apoptosis [47, 48]. The PAM pathway is activated in most tumors, including human PCa, and contributes to tumor development by promoting nutrient metabolism, angiogenesis, cell proliferation and motility. Activation of this pathway in PCa cells is induced by mutations or deletions in PTEN and is associated with resistance to anticancer drugs [48, 49]. AKT, a downstream effector of PI3K, upregulates androgen receptor activity at the transcriptional level and activates its downstream transcription factors, including FOXO, thereby promoting PCa development and progression [49, 50]. mTOR, another downstream factor of PI3K that is hyperactivated in many tumors, maintains cellular homeostasis by sensing nutrient signals and regulating metabolism, growth and proliferation [51, 52], highlighting PAM signaling as a potential target for PCa treatment. In contrast, downregulation of the ROS-mediated PAM pathway by cisplatin was efficiently blocked by platycodin D [21], alleviating cell and, tissue damage due to oxidative stress in T2DM and macrophage inflammation models [19, 20]. In this study, the PAM pathway was markedly inactivated in platycodin D-treated PC-3 cells, as reflected by the reduced phosphorylation of PI3K, AKT and mTOR (Fig. 6). This observation aligns with previous studies in other cancer cell types [15–18]. Moreover, the activation of PAM signaling and the degradation of PARP were restored by NAC pretreatment. Corroborating these results, NAC blocked ROS generation and effectively reversed the growth inhibitory effects and apoptosis induced by platycodin D. Conversely, a PI3K-specific inhibitor potentiated platycodin D-induced cytotoxicity, indicating that these effects in PC-3 cells were, at least in part, PAM signaling-dependent (Fig. 7).

Our results indicate that elevation of ROS levels is a key event in the induction of apoptosis by platycodin D in PC-3 cells, serving as an upstream regulator of the inactivation of the PAM signaling (Fig. 8). While mitochondria are the main

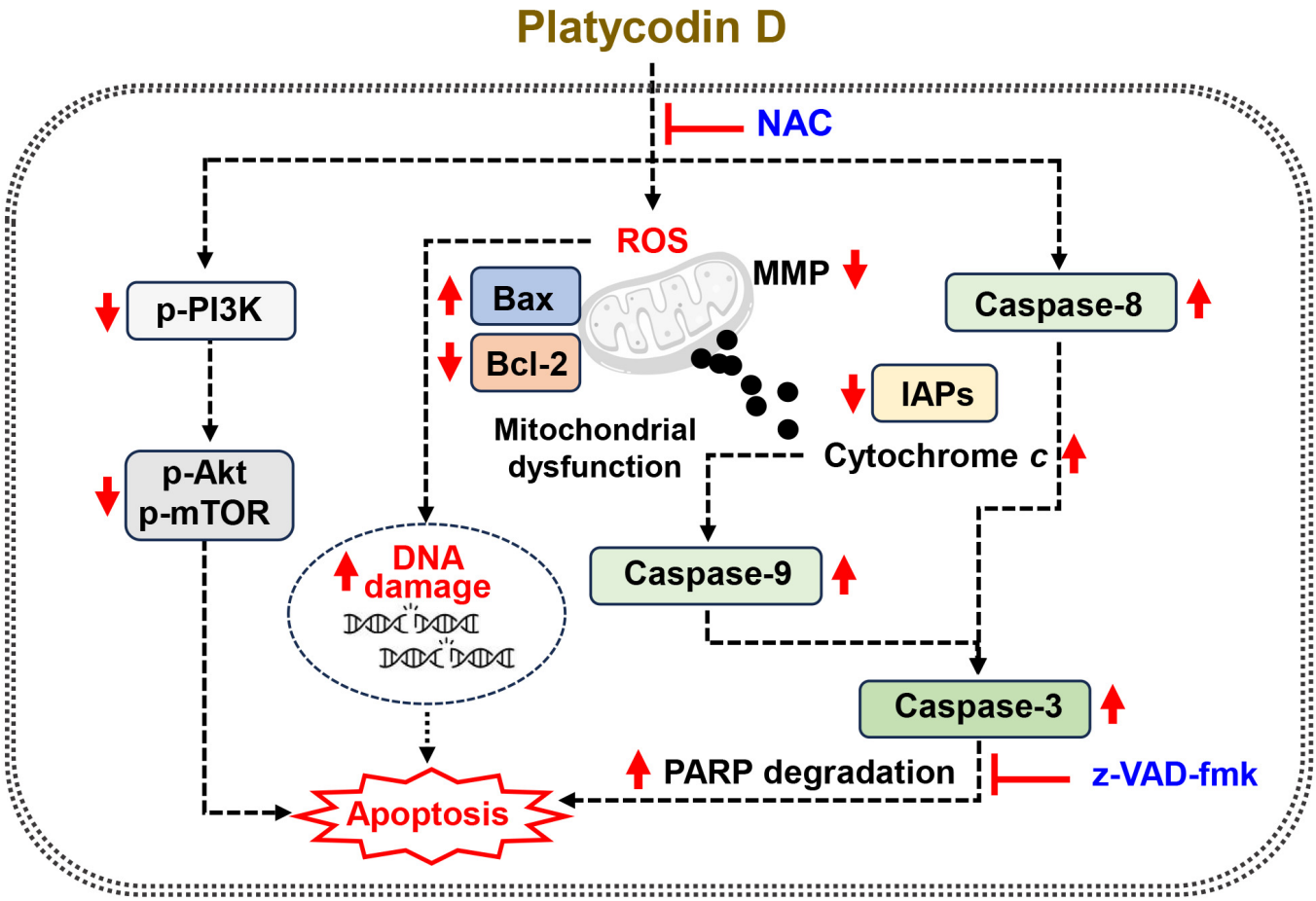

FIGURE 8. Schematic of the proposed mechanism of platycodin D-induced cytotoxicity in PC-3 cells. The anticancer effects of platycodin D are proposed to involve excessive mitochondrial ROS generation, leading to mitochondrial dysfunction and inactivation of the PAM signaling pathway. NAC: N-acetyl-L-cysteine; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin; ROS: reactive oxygen species; MMP: mitochondrial membrane potential; IAP: inhibitor of apoptosis protein; PARP: poly (ADP-ribose) polymerase; z-VAD-fmk: carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

intracellular source of ROS, excessive accumulation of ROS mediates mitochondrial dysfunction as ROS are highly reactive towards various macromolecules involved in the electron transport chain [53, 54]. However, since ROS generation is associated with multiple pathways of redox homeostasis and cellular signaling, in addition to the mitochondrial damage associated with aerobic respiration [53, 55], further detailed investigation into the specific origins of ROS production is warranted. It is well known that the genetic background of tumors influences their sensitivity to anticancer chemotherapy and is a major factor in determining the selectivity of anticancer therapy. Consequently, co-targeting specific signaling pathways and associated pathways is essential to improve the efficacy of anticancer therapies in clinical settings [56, 57]. Zhou *et al.* [29] found that the sensitivity of PCa cells to platycodin D varies depending on their genetic background. They used three cell lines (PC-3, LNCaP and DU145) and observed that PC3 cells were more sensitive to platycodin D than the other two. Specifically, the PC-3 cells used in this study are androgen-independent and PTEN-null, DU145 cells are androgen-independent and PTEN-positive, and LNCaP cells are androgen-responsive and PTEN-negative [58]. Therefore, while other genetic differences should be considered, exploring the role of androgens and PTEN in regulating the PAM pathway, which is targeted by platycodin D in PCa cells, presents an interesting avenue for future research.

5. Conclusions

Our results demonstrate that platycodin D induces DNA damage in human PCa PC-3 cells by increasing ROS production, decreasing cell survival and inducing apoptosis. However, these effects of platycodin D were significantly diminished by ROS scavenging, indicating that platycodin D exerts ROS-dependent anticancer activity in PC-3 cells. Furthermore, our data show that platycodin D inhibits the ROS-mediated PAM pathway, thereby promoting apoptosis and suppressing cell proliferation. Collectively, these findings suggest that ROS generation is upstream of platycodin D-induced apoptosis and PAM pathway inactivation in PC-3 cells (Fig. 8).

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

YHC—designed the study and carried them out; supervised the data collection, analyzed the data, interpreted the data; prepared the manuscript for publication and reviewed the draft of the manuscript; read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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