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Induction of apoptosis through inactivation of ROS-dependent PI3K/Akt signaling pathway by platycodin D in human bladder urothelial carcinoma cells

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Abstract. Platycodin D (PD) is a triterpenoid saponin, a major bioactive constituent of the roots of *Platycodon grandiflorum*, which is well known for possessing various pharmacological properties. However, the anti-cancer mechanism of PD in bladder cancer cells remains poorly understood. In the current study, we investigated the effect of PD on the growth of human bladder urothelial carcinoma cells. PD treatment significantly reduced the cell survival of bladder cancer cells associated with induction of apoptosis and DNA damage. PD inhibited the expression of inhibitor of apoptosis family members, activated caspases, and induced cleavage of poly (ADP-ribose) polymerase. PD also increased the release of cytochrome *c* into the cytoplasm by disrupting the mitochondrial membrane potential while upregulating the expression ratio of Bax to Bcl-2. The PD-mediated anti-proliferative effect was significantly inhibited by pre-treatment with a pan-caspase inhibitor, but not by an inhibitor of necroptosis. Moreover, PD suppressed the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway, and the apoptosis-inducing effect of PD was further enhanced by a PI3K inhibitor. In addition, PD increased the accumulation of reactive oxygen species (ROS), whereas N-acetyl cysteine (NAC), an ROS inhibitor, significantly attenuated the growth inhibition and inactivation of the PI3K/

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Akt/mTOR signaling caused by PD. Furthermore, NAC significantly suppressed apoptosis, DNA damage, and decreased cell viability induced by PD treatment. Collectively, our findings indicated that PD blocked the growth of bladder urothelial carcinoma cells by inducing ROS-mediated inactivation of the PI3K/Akt/mTOR signaling.

Key words: Platycodin D — Apoptosis — ROS — PI3K/Akt/mTOR

Introduction

Bladder cancer is one of the very common types of urinary system cancer commonly found in the bladder mucosa. The incidence of bladder cancer has increased significantly worldwide over the past decade, with the highest rates in the United States and Europe (Mahdavifar et al. 2016; Fankhauser and Mostafid 2018). Transurethral resection is generally performed for the treatment of these patients, but the recurrence rate of tumors after surgery has not improved (Preston et al. 2015; Fankhauser and Mostafid 2018). Although various chemotherapies are being applied to suppress the recurrence rate and improve the survival rate of these patients, these drugs have toxic side effects and serious complications (Godwin et al. 2018; Harraz et al. 2019). Therefore, there is an urgent need to discover new agents that are effective and safe with fewer side effects that can selectively suppress the growth of bladder cancer cells. In this respect, natural phytochemicals are coming into the spotlight due to their low cost, low toxicity, and low hostility in bladder cancer prevention and therapy (Xia et al. 2021). Recently, several medical plants and phytochemicals, including *Radix Sophorae Flavescentis*, *Daphne genkwa, Tripterygium wilfordii*, and flavonoids, steroids, alkaloids and quinone compounds from these natural plants have been reported to have anti-bladder cancer effect through apoptosis inducing (Jo et al. 2020; Xia et al. 2021). Apoptosis, also known as programmed cell death, is a precisely and essential regulated mechanism for regulating cell and tissue homeostasis and organism function in eukaryotic cells. In particular, one of the hallmarks of constantly dividing cancer cells is escape from apoptosis (Hanahan and Weinberg 2011; Kiraz et al. 2016). Therefore, inducing apoptosis of cancer cells in all types of cancer treatment, including bladder cancer, is recognized as the most representative strategy. Actually, some phytochemicals have been developed as first-line anticancer drugs, such as paclitaxel and vincristine, and have acceptable clinical evidence that supports their anticancer efficacy by promoting cell apoptosis and reducing cell proliferation (Khan et al. 2020; Xia et al. 2021).

Platycodin D (PD), a type of triterpenoid saponin, is the main active component of the root of *Platycodon grandiflorum* (Jacq.) A.DC., which has been widely used in prescriptions for the prevention and treatment of various diseases in several countries in East Asia including Korea, China, and Japan (Zhang et al. 2015; Ji et al. 2020). In support of these uses, a number of pharmacological effects of PD have been reported, including anti-inflammatory, antioxidant, anti-atherogenic, hepatoprotective, anti-obesity, and immunological adjuvant activities (Hwang et al. 2013; Khan et al. 2016; Wang et al. 2019; Zhang et al. 2020). Previous studies also demonstrated that PD exhibited anti-proliferative effects by inducing cell cycle arrest and apoptosis in many types of cancer cells (Shin et al. 2009; Chun and Kim 2012; Yu and Kim 2012; Xu et al. 2014; Zhao et al. 2015; Khan et al. 2016; Seo et al. 2018; Zhang et al. 2020). Although Chen et al. (2020, 2021) recently reported that PD inhibited the proliferation of human bladder cancer cells through induction of apoptosis; the underlying mechanisms involved in apoptosis are still unclear. Therefore, in the present study, we investigated the effect of PD on apoptosis in human bladder urothelial carcinoma cells and identified the mechanism.

Materials and Methods

Chemicals and reagents

RPMI 1640 medium, fetal bovine serum (FBS) and antibiotic mixtures were obtained from WelGENE Inc. (Gyeongsan, Republic of Korea). PD, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 4',6'-diamidino-2-phenylindole (DAPI), N-acetyl-L-cysteine (NAC), *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) and necrostain-1 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The genomic DNA isolation kit and LY294002 were purchased from Biovision (Milpitas, CA, USA) and AG Scientific, Inc. (San Diego, CA, USA), respectively. The fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) double staining kit was obtained from Becton Dickinson (San Jose, CA, USA). 2',7'-dichlorofluorescein-diacetate (DCF-DA) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Caspase enzyme-linked immunosorbent assay (ELISA) kits were provided from R&D Systems, Inc. (Minneapolis, MN, USA). The mitochondrial fractionation kit and polyvinylidene difluoride (PVDF) membranes were purchased from Active

Motif, Inc. (Carlsbad, CA, USA) and (Schleicher & Schuell, Keene, NH, USA), respectively. The comet assay reagent kit was obtained from Trevigen (Gaithersburg, MD, USA). The EpiQuik 8-hydroxydeoxyguanosine (8-OHdG) DNA damage quantification kit was purchased from Epigentek (Cambridge, UK). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Abcam, Inc. (Cambridge, MA, UK) and Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescent (ECL) reagent were provided from Santa Cruz Biotechnology, Inc. and Thermo Fisher Scientific, respectively.

Cell culture and PD treatment

Human bladder urothelial carcinoma cell lines (EJ, UMUC-3 and T24) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium containing 10% FBS and antibiotic mixtures in a humidified incubator at 5% CO_2 and 37°C. In order to treat PD to cells, it was dissolved in DMSO and then diluted in a medium.

Cell viability assay

To measure cell viability, the MTT assay was performed, as described previously (Hwangbo et al. 2020). In brief, cells were treated with various concentrations of PD for 48 h or pre-treated with 50 μM z-VAD-fmk 200 μM necrostain-1, 10 μM LY294002, or 10 mM NAC for 1 h and then treated with 30 μM PD. The cells were then incubated in a medium containing 50 μg/ml of MTT at 37°C. After 3 h, the medium from each well was removed, DMSO was added, and the reaction was performed at room temperature (RT) for 10 min to dissolve the formazan crystals. Absorbance was immediately measured at 540 nm with an ELISA microplate reader (Becton Dickinson).

Quantitative analysis of apoptosis by flow cytometry

To quantitatively analyze the induction of apoptosis, flow cytometry analysis by annexin V and PI staining was performed, according to the manufacturer's procedure. In brief, the treated cells were washed with phosphate-buffered saline (PBS) and then stained with annexin V/PI solution for 30 min. Subsequently, annexin V-positive cells were counted as apoptotic cell population using a flow cytometer, as previously described (Ojima et al. 2021).

Detection of nuclear morphological changes

DAPI staining was used to investigate whether apoptosis was induced by examining the morphological changes of the nucleus. Briefly, treated cells were washed with PBS, fixed using 3.7% paraformaldehyde at RT for 10 min, and then stained with DAPI solution (1 μg/ml) in the dark for 10 min, as previously described (Choi 2021). After washing the cells again with PBS, the morphology of the nucleus was observed using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Western blot analysis

The treated cells were lysed to extract whole proteins, as previously described (Choi et al. 2021). The cytoplasmic and mitochondrial proteins were extracted using a mitochondrial isolation kit, according to the manufacturer's instructions. To separate equal amounts of proteins from individual lysates, they were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and then transferred to PVDF membranes. The membrane was then blocked with 3% bovine serum albumin at room temperature for 30 min, and specific primary antibodies were added to the membranes, incubated overnight at 4°C, and subsequently probed with appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, ECL reagent was used to visualize the proteins of interest, according to the manufacturer's protocol.

Caspase activity assay

The activities of caspases of cells cultured under various conditions were detected using ELISA assay kits, according to the kit instructions. Briefly, after lysis of cells using the lysis buffer provided in the kit, the supernatants containing the same amount of protein were collected and mixed with reaction buffer for 2 h at 37°C. After the reaction, the optical density of each sample was measured by absorbance at 405 nm using an ELISA microplate reader and presented as relative values, as previously described (Liang et al. 2020).

Mitochondrial membrane potential (MMP) assay

JC-1 staining was performed to measure MMP, according to the manufacturer's instruction. Briefly, treated cells were collected and stained with 10 μM JC-1 at 37°C for 30 min. Cells were washed with PBS and MMP values were measured at excitation and emission wavelengths of 488 and 575 nm using a flow cytometer (Hwangbo et al. 2020).

Reactive oxygen species (ROS) generation assay

The level of ROS accumulated in cells was investigated using DCF-DA reagent, as previously described (Bae et al. 2020). Briefly, cells cultured for 1 h in PD-treated medium in the presence or absence of 10 mM NAC were collected and react-

ed with 10 μM DCF-DA. After the 20 min reaction, the level of ROS generation in each sample was immediately measured using flow cytometry at excitation and emission wavelengths of 480 and 520 nm. The cells stained with DCF-DA were also observed under a fluorescence microscope (Lee et al. 2020).

Comet assay

The comet assay was applied to analyze DNA damage using a Comet Assay Reagent Kit based on the manufacturer's protocol. Briefly, following treatment, the cells were detached from the wells and mixed with 0.5% low-melting-point agarose at 37°C. The cells are spread out completely on frosted-glass microscope slides, pre-coated with 1% normal melting agarose, cooled, and coagulated using an ice pack for 5 min. The slides were immersed in the lysis solution at 4°C for 1 h and then placed in a gel electrophoresis device for 30 min to allow the DNA to unwind, followed by electrophoresis for 30 min, as previously described (Choi 2021). Subsequently, each slide was washed with the neutralization buffer provided in the kit, stained with 20 μg PI, and microscopic images then captured under a fluorescence microscope.

8-OHdG assay

For the quantitative evaluation of DNA damage due to oxidative stress, the HT 8-oxodG ELISA Kit was used to measure the level of 8-OHdG. Briefly, following treatment, whole genomic DNA was extracted using a DNA purification kit, according to the manufacturer's protocol, and quantified with the NanoDrop^{TM} 2000 (Thermo Scientific, Wilmington, DE, USA). The extracted DNA was digested sequentially with DNase I, and the levels of 8-OHdG were detected by fluorescence at 450 nm, according to the kit instructions (Park et al. 2019).

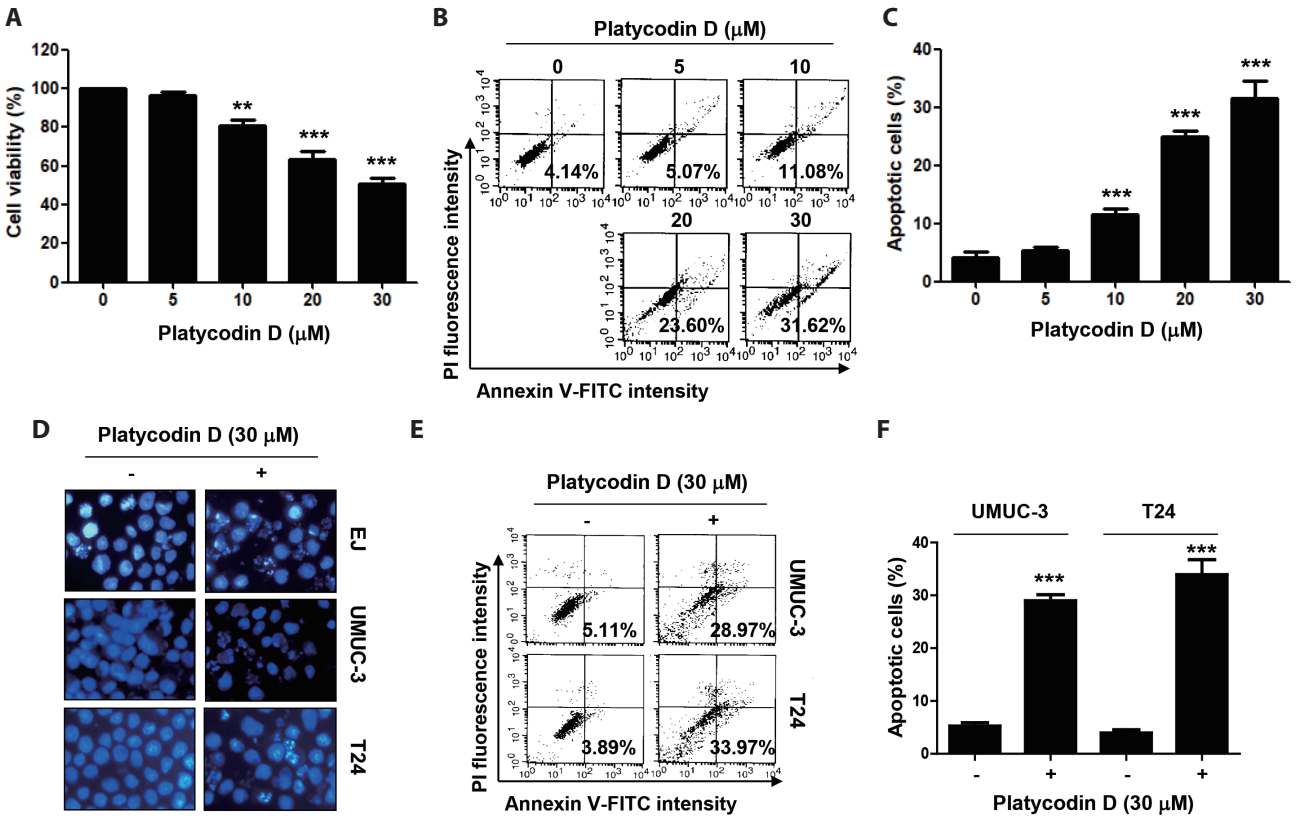

Figure 1. Platycodin D inhibits cell viability and induces apoptosis in human bladder urothelial carcinoma cells (EJ, UMUC-3 and T24). Cells were treated with different doses of platycodin D for 48 h. **A.** Cell viability of platycodin-treated EJ cells was measured by the MTT assay. **B.** Representative profiles of flow cytometry results obtained after annexin V and PI double staining of EJ cells treated with different concentration of platycodin. **C.** Quantitative analysis of apoptosis-induced EJ cells. The frequencies of cells induced apoptosis were expressed as percentages of the numbers of annexin V-positive cells. **D.** Nuclei of DAPI-stained cells were observed under a fluorescence microscope. **E.** Representative profiles of flow cytometry results obtained after annexin V/PI staining of UMUC-3 and T24 cells treated with 30 μM platycodin. **F.** Quantitative analysis of apoptosis-induced UMUC-3 and T24 cells. Data were presented as mean ± SD of three independent experiments (** $p < 0.01$ and *** $p < 0.001$ *vs.* untreated cells).

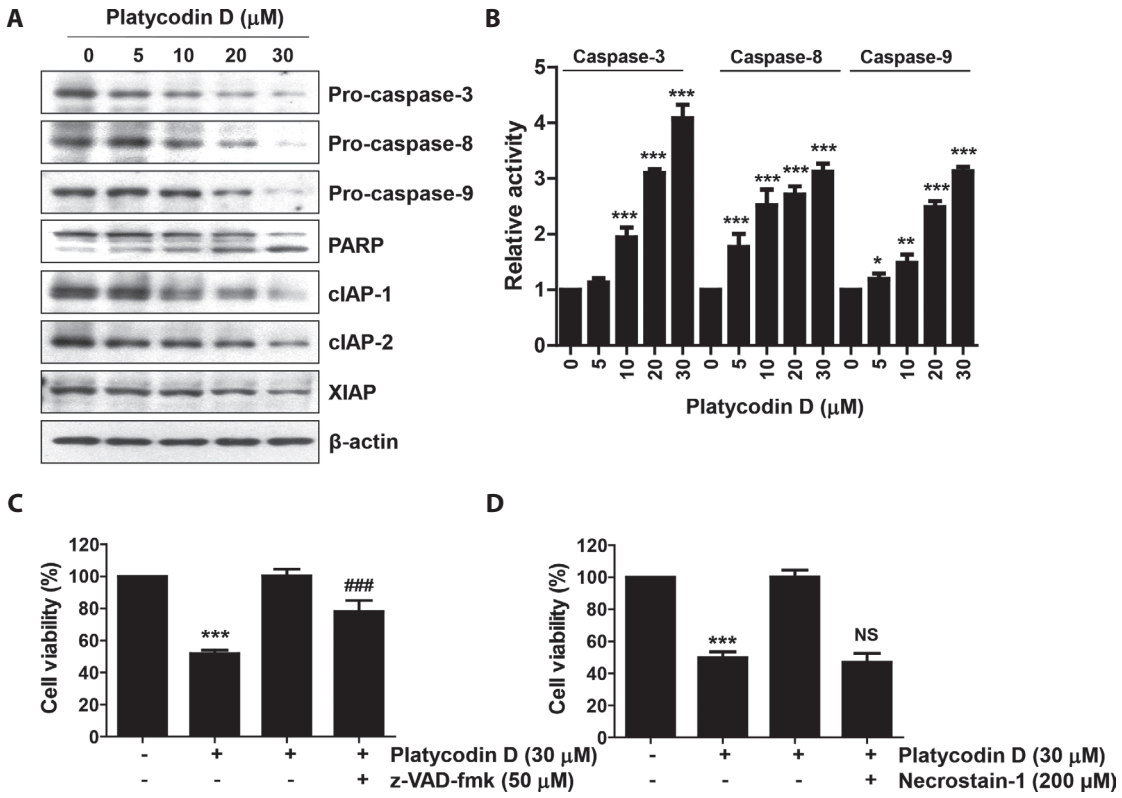

Figure 2. Platycodin D activated caspases in EJ cells. Cells were treated with the indicated concentrations of platycodin for 48 h. **A.** Western blot analysis for the indicated proteins and β-actin was used as a loading control. **B.** Colorimetric ELISA assay kits were used to measure the activity of caspases. Cell viability of cells exposed to 30 μM platycodin for 48 h in the presence or absence of 50 μM z-VAD-fmk (**C**) or 200 μM necrostein-1 (**D**) for 1 h was measured by MTT assay. Data was presented as the mean ± SD (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* untreated cells; ### $p < 0.001$ *vs.* 30 μM platycodin-treated cells). NS, not significant compared to 30 μM platycodin-treated cells.

Statistical analysis

GraphPad Prism 5.03 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. All experimental values are presented as the mean ± standard deviation (SD). The statistical analysis examined differences between the groups through a one-way analysis of variance followed by Tukey's post-test, and *p*-values of < 0.05 were considered to represent a statistically significant difference.

Results

PD inhibits cell viability and induces apoptosis in human bladder cancer cells

To assess the growth inhibitory effect of PD in human bladder cancer EJ cells, cells were treated with different concentrations of PD for 48 h and then cell viability was measured by the MTT assay. Figure 1A shows that PD significantly decreased cell viability with increasing treatment concentrations. Therefore, we investigated whether the proliferation inhibition of PD-treated EJ cells was associated with apoptosis induction and found that PD induced apoptosis in the results using annexin V/PI staining in a concentration-dependent manner (Fig. 1B and C). In addition, condensed and fragmented nuclei were observed in PD-treated cells through DAPI staining, indicating that the growth inhibition of EJ cells by PD was related to the apoptosis induction (Fig. 1D). We next examined whether PD induces apoptosis in other bladder cancer cells (UMUC-3 and T24) and found that it induced apoptosis with a similar trend in the two cell lines investigated (Fig. 1D–F).

PD activates caspases in human bladder cancer EJ cells

We chose EJ cells to identify the mechanism of apoptosis and found that the expression of Fas ligand (FasL) was slightly enhanced by PD in a dose-dependent manner, but the expression of death receptor 4 was not changed. Furthermore, PD reduced the expression of the pro-forms of caspases, including caspase-3, -8, and -9, and induced

the degradation of poly (ADP-ribose) polymerase (PARP), one of the substrates of the activated effector caspase (Fig. 2A), which was associated with inhibition of the expression of the inhibitor of apoptosis (IAP) family proteins such as cIAP-1, cIAP2, and XIAP. Consistent with Western blotting results, the caspase activities were also greatly increased by PD (Fig. 2B). Therefore, we further investigated whether PD-induced apoptosis was caspase-dependent using the pan-caspase inhibitor, z-VAD-fmk. As indicated in Figure 2C, pre-treatment with z-VAD-fmk markedly rescued cell viability in the PD-treated cells. However, pre-treatment with a necroptosis inhibitor, necrostain-1, had no significant effect on the inhibition of cell viability by PD (Fig. 2D), meaning that the decreased proliferation of EJ cells by PD was caused by apoptosis, not necroptosis.

PD increases mitochondrial dysfunction with altered expression of Bcl-2 family members in human bladder cancer EJ cells

We further assessed the expression of mitochondrial pathway-associated proteins and the levels of MMP in PD-treated cells. As shown in Figure 3A, PD stimulation up-regulated the expression of pro-apoptotic Bax protein, but the expression of anti-apoptotic Bcl-2 was downregulated in a concentration-dependent manner. In addition, cytochrome *c* release into the cytoplasm was increased in PD-treated cells (Fig. 3B). Furthermore, flow cytometry analysis showed that PD triggered the loss of MMP (Fig. 3C). And, in fluorescence microscopy observation of JC-1 staining, red fluorescence, a signal of preserved MMP, was strongly observed in untreated control cells, whereas the green fluorescence intensity was slightly higher in PD-treated cells (Fig. 3D), indicating that the mitochondrial membrane was depolarized.

PD inactivates the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway in human bladder cancer EJ cells

To evaluate the role of the PI3K/Akt/mTOR signaling pathway in the inhibition of EJ cell proliferation by PD, the changes in expression of PI3K and its downstream components Akt and mTOR were measured. As shown in Figure

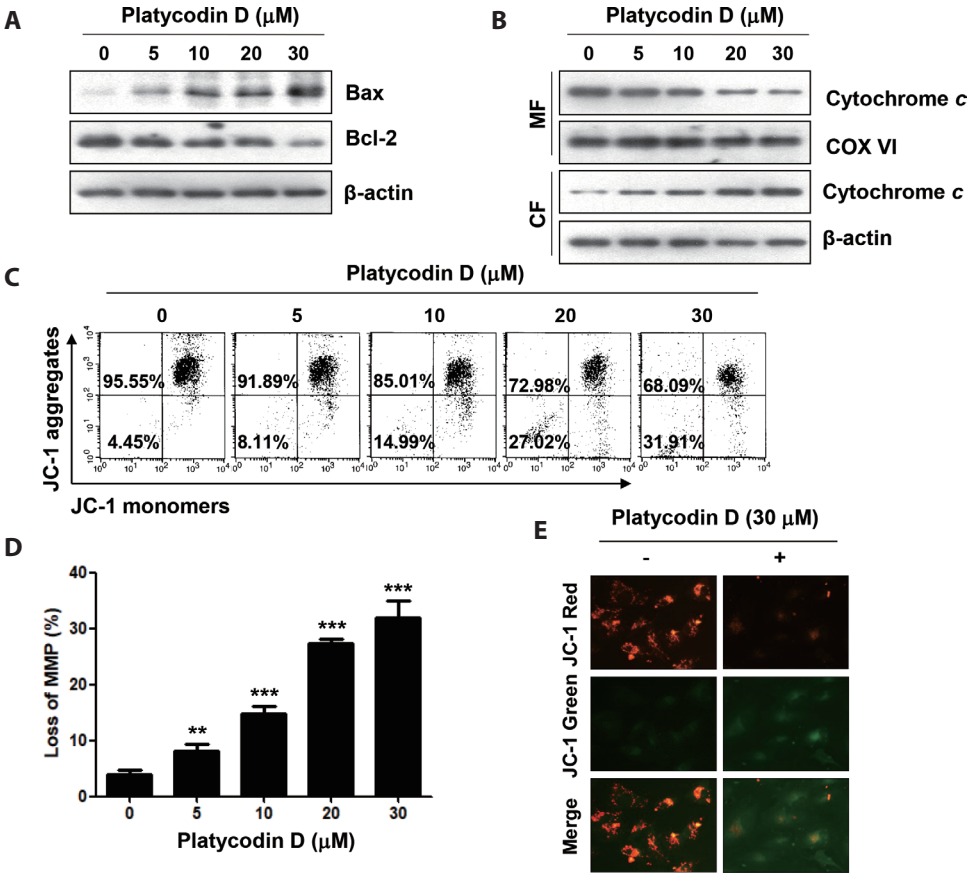

Figure 3. Platycodin D altered the expression of Bcl-2 family proteins and induced cytoplasmic release of cytochrome *c* and loss of MMP in EJ cells. Cells were treated with various concentrations of platycodin (5–30 μM) for 48 h. **A.** Western blot analysis of Bcl-2 family proteins (β-actin was used as an internal control). **B.** Cytochrome *c* expression was analyzed from isolated mitochondrial and cytoplasmic proteins. Cytochrome oxidase subunit VI (COX VI) and β-actin were included as loading controls, respectively. **C.** Cells were stained with JC-1 dye and then flow cytometry was performed to determine the extent of MMP loss. **D.** Quantitative analysis of loss of MMP in EJ cells. Data was presented as the mean ± SD (** $p < 0.01$ and *** $p < 0.001$ *vs.* untreated cells). **E.** The fluorescence intensity after staining with JC-1 dye. Red fluorescence of the JC-1 aggregates indicates high MMP, whereas green fluorescence of the JC-1 monomers indicates low MMP. MF, mitochondrial fraction; CF, cytosolic fraction; MMP, mitochondrial membrane potential. (For color figure, see online version of the manuscript).

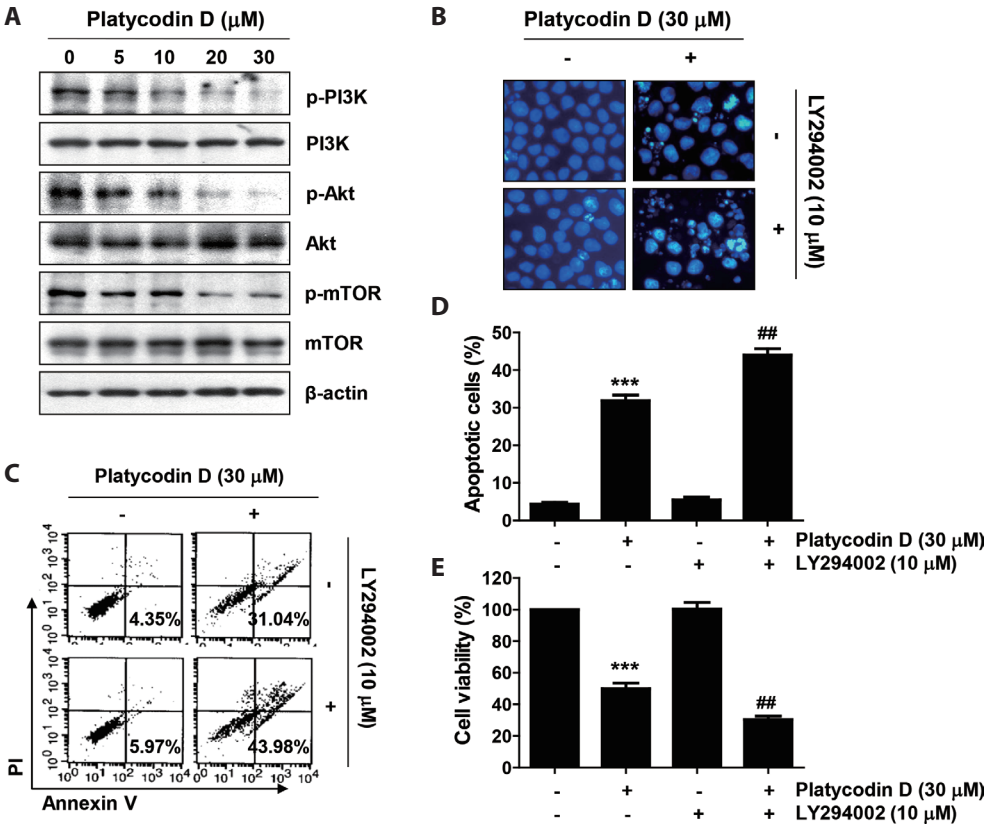

Figure 4. Platycodin D induced the inactivation of the PI3K/Akt/mTOR signaling pathway in EJ cells. A. Western blot analysis for the indicated proteins in cells cultured in media containing various concentrations of platycodin D for 48 h. **B.** Nuclei of DAPI-stained cells were observed under a fluorescence microscope. **C.** The frequencies of cells induced apoptosis were expressed as percentages of the numbers of annexin V-positive cells. **D.** Number of apoptotic cells per slide was estimated by counting apoptotic cells in five different fields. **E.** Cell viability of cells cultured uder different conditions measured using the MTT assay. Data was presented as the mean ± SD (*** $p < 0.001$ *vs.* untreated cells; ## $p < 0.01$ *vs.* 30 μM platycodin D-treated cells).

4A, the expression levels of phosphorylated (p)-PI3K, p-Akt, and p-mTOR were gradually reduced with increasing PD concentrations without any change in the total protein, suggesting that PD suppressed the PI3K/Akt/mTOR signaling activity. Therefore, we investigated the effect of combination treatment of PD with LY294002, a PI3K inhibitor, on the anticancer activity of PD. Our results showed a more significant increase in apoptosis and a further decrease in cell viability in EJ cells co-treated with both compounds compared to PD alone, as shown by the DAPI staining, flow cytometry, and MTT assay results (Fig. 4B–D). In addition, combination treatment of PD with LY294002 significantly up-regulated the expression of Bax and down-regulated the expression of Bcl-2 compared with PD alone (Fig. 4F).

PD induces intracellular ROS generation and DNA damage in human bladder cancer EJ cells

Next, we evaluated whether ROS production is involved in the anticancer activity of PD. As shown in Figure 5A and B, the flow cytometry by DCF-DA staining showed that ROS levels increased greatly within 1 h of PD treatment and then gradually decreased thereafter. However, ROS generation by PD was significantly diminished by the ROS scavenger, NAC, and the results of fluorescence microscopy were also consistent with these results (Fig. 5C). We also investigated whether PD-induced EJ cell apoptosis was related to DNA damage. The results indicated that the expression of p-γH2AX was markedly increased after PD stimulation in a concentration-dependent manner without significant changes in the total protein expression (Fig. 6A). However, NAC significantly attenuated the increased expression of p-γH2AX following PD treatment (Fig. 6B). Moreover, the elevated levels of 8-OHdG and tail moment of the comet assay following PD treatment were markedly diminished after NAC treatment (Fig. 6C and D).

Inactivation of the PI3K/Akt/mTOR signaling pathway by PD is ROS-dependent in human bladder cancer EJ cells

Based on the above observation that PD promoted intracellular ROS production and inactivation of the PI3K/Akt/mTOR signaling pathway, we evaluated the role of ROS in PD-induced apoptosis in EJ cells. As shown in Figure 7A, when ROS production was blocked by NAC, the levels of p-PI3K and p-Akt were maintained at control levels in PD-treated cells, and PARP degradation was not induced. NAC pre-treatment also significantly restored PD-induced apoptosis, as observed in the results of DAPI staining and flow cytometry (Fig. 7B–D). Consistent with these results,

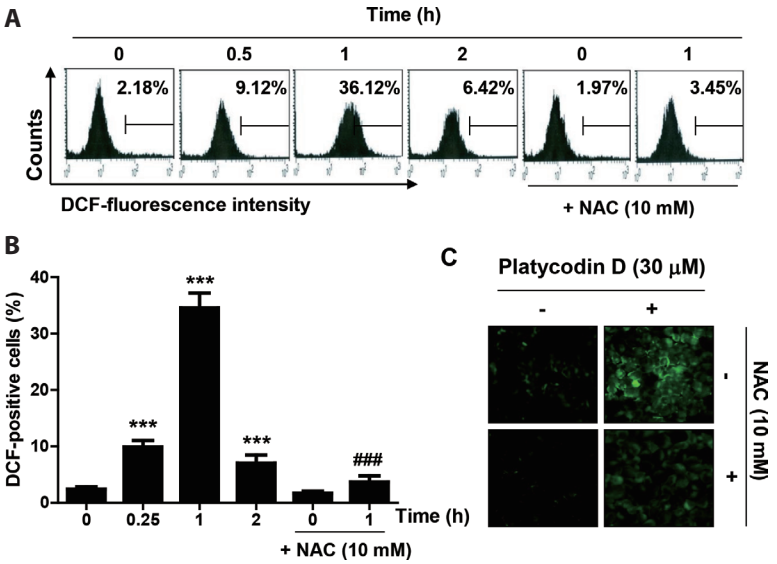

Figure 5. Platycodin D increased ROS production in EJ cells. Cells were cultured in media containing 30 μM platycodin D for the indicated times, or pre-treated with 10 mM NAC for 1 h, followed by exposure to 30 μM platycodin D for an additional 1 h. **A.** Representative profiles for ROS generation measured by flow cytometry after staining with DCF-DA. **B.** Quantitative analysis of ROS generation. Data were presented as the mean ± SD (*** $p <$ 0.001 *vs.* untreated cells; ### $p < 0.001$ *vs.* 30 μM platycodin D-treated cells). **C.** Representative fluorescence micrographs depicting ROS generation and accumulation in EJ cells.

the PD-induced reduction in cell viability was significantly protected by inhibiting ROS generation (Fig. 7E). Furthermore, pre-treatment of NAC markedly relieved the increasing of Bax/Bcl-2 ratio following by PD (Fig. 7F). These results demonstrated that ROS triggered apoptosis as an upstream initiator in the induction of inactivation of the PI3K/Akt/mTOR pathway in PD-treated EJ cells.

Discussion

Among the two main pathways inducing apoptosis, the extrinsic pathway is initiated by activation of caspase-8 according to the binding of death ligands and DRs (Saki and Prakash 2017; Pfeffer and Singh 2018). Subsequently, activation of the initiator caspase-8 can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 (Boice and Bouchier-Hayes 2020). In contrast, activation of the intrinsic pathway involves mitochondrial dysfunction due to altered expression of Bcl-2 family proteins caused by various apoptosis-inducing stimuli (Kiraz et al. 2016; Prates et al. 2018). Along with this, apoptogenic factors like cytochrome *c*, as a consequence of increased mitochondrial permeability, must be released from the mitochondria into the cytoplasm to induce the activation of caspase-9. Therefore, caspase-8 and -9 are broadly classi-

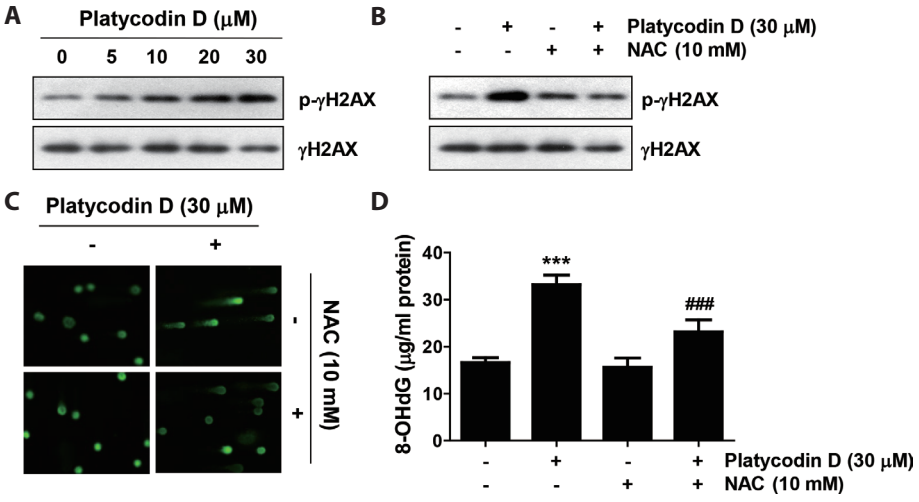

Figure 6. Platycodin D induced ROS-dependent DNA damage platycodin D in EJ cells. Cells were cultured in media containing the indicated concentrations of platycodin D for 48 h or pre-treated with 10 mM NAC for 1 h, followed by exposure to 30 μM platycodin D for an additional 48 h **A.** Protein expressions of p-γH2AX and γH2AX was measured by immunoblotting analysis. **B.** Western blot analysis of p-γH2AX and γH2AX were evaluated in presence of 10 mM NAC. **C.** The comet assay was evaluated and representative fluorescence micrographs of comet assay results are presented. **D.** 8-OHdG levels were measured using an ELISA kit. Each data was presented as the mean ± SD (*** $p < 0.001$ *vs.* untreated cells; ### $p < 0.001$ *vs.* 30 μM platycodin D-treated cells).

fied as initiator caspases of the two apoptosis pathways, and activate executor caspases to complete apoptosis through the degradation of matrix proteins necessary for cell survival, including PARP (Birkinshaw and Czabotar 2017; Edlich 2018). However, proteins belonging to the IAP family have the ability to bind directly to caspases and inhibit their activity (Cong et al. 2019; Kumar et al. 2020). Meanwhile, numerous studies have been investigated the apoptotic effect of PD on the extrinsic and intrinsic pathway in various cell lines (Yu and Kim 2010; Li et al. 2014; Xu et al. 2014; Zhou et al. 2015; Khan et al. 2016). Additionally, PD has been reported extrinsic apoptosis by inducing the expression of FasL and proteolytic cleavage of caspase-3, -8, -9 and PARP in gastric cancer cells (Chun et al. 2013). Furthermore, PD has markedly suppressed the Bcl-2/Bax ration and increase caspase-3 and -9 activation in several cell lines (Chun et al. 2013). In this study, we also found that PD effectively induced apoptotic cell death in human bladder urothelial carcinoma cells. Importantly, the concentrations of PD used in the present experiments did not significantly affect the viability of normal cells (Khan et al. 2016; Chen et al. 2021), indicating that PD may selectively induce apoptosis in cancer cells. We demonstrated that PD activated caspase-9 as well as caspase-8, inhibited the expression of IAP-family proteins, and promoted mitochondrial dysfunction in EJ cells, accompanied by enhancement of the Bax/Bcl-2 expression ratio, cytoplasmic release of cytochrome *c*, and loss of MMP. In addition, increased caspase-3 activity and PARP degradation were observed in PD-treated cells. Moreover, our finding showed that PD induced upregulation of FasL, while did not affect the expression of DR4, indicating that extrinsic pathway was partially involved in PD-induced apoptosis. However, the anticancer activity by PD was significantly inhibited in the presence of a pan-caspase inhibitor, but there was no significant difference by the inhibitor of necroptosis indicating that PD induced apoptosis through simultaneous activation of extrinsic and intrinsic pathways in a caspase-dependent manner, not by necroptosis.

Apoptosis inducing pathway are regulated by the interaction of various signaling pathways and apoptosis regulators, which can be switched by controlling these key regulatory steps (Fouad and Aanei 2017; Pfeffer and Singh 2018). Among these, the PI3K/Akt/mTOR pathway is involved in a variety of intracellular processes such as cell division, proliferation, differentiation and cell death (Mayer and Arteaga 2016; Lien et al. 2017). Abnormal activation of this pathway is known to play a key role in the onset of various types of human tumors, including bladder cancer (Houédé and Pour-

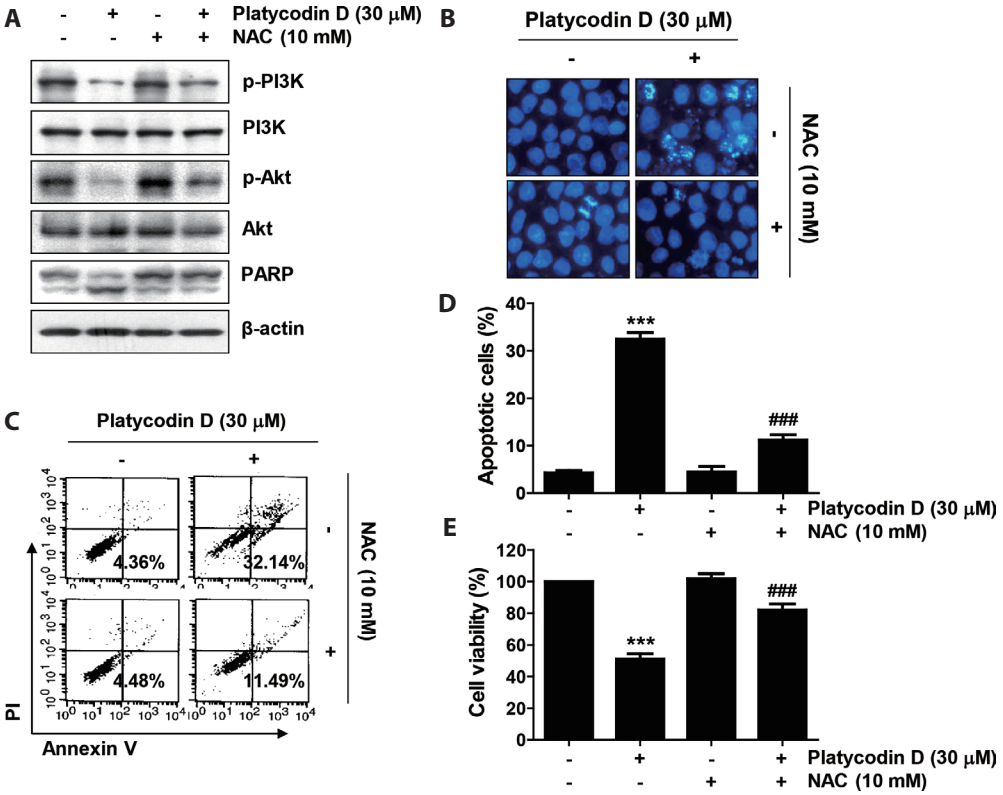

Figure 7. Platycodin D promoted ROS-mediated inactivation of the PI3K/Akt/mTOR signaling in EJ cells. Cells were either treated with 30 μM platycodin D for 48 h, or pretreated with 10 mM NAC for 1 h, followed by exposure to 30 μM platycodin D for an additional 48 h. **A.** Western blot analysis for the indicated proteins were presented (β-actin was included as a loading control). **B.** Nuclei of DAPI-stained cells were observed under a fluorescence microscope. **C.** Representative profiles of flow cytometry results obtained after annexin V/PI staining were presented. **D.** The frequencies of cells induced apoptosis were expressed as percentages of the numbers of annexin V-positive cells. **E.** Cell viability of cells cultured under different conditions was measured through the MTT assay. Each data was presented as the mean ± SD (*** $p < 0.001$ *vs.* untreated cells; ### $p < 0.001$ *vs.* 30 μM platycodin D-treated cells).

quier 2015; Sathe and Nawroth 2018). When Akt and mTOR, downstream factors of PI3K, are phosphorylated by activated PI3K, they inhibit the induction of apoptosis by blocking the initiation of the caspase cascade by phosphorylating caspases. In addition, they enhance the activity of anti-apoptotic proteins belonging to the Bcl-2 family, enabling the survival and proliferation of cancer cells (Hoxhaj and Manning 2020). Ultimately, PI3K and its modulators may be attractive targets in cancer treatment, as they contribute to chemotherapeutic resistance, which induces cancer cell apoptosis (Mayer and Arteaga 2016; Lien et al. 2017). Similar to the results of several previous studies (Chun and Kim 2013; Xu et al. 2014; Zhao et al. 2015; Seo et al. 2018; Chen et al. 2021), we also found that PD reduced the levels of phosphorylated PI3K, Akt, and mTOR, meaning that the PI3K/Akt/mTOR signaling was inactivated in PD-treated cells. Furthermore, an inhibitor of PI3K, LY294002, markedly increased the apoptotic potential of PD and further suppressed cell viability, supporting our conclusion that PD-induced EJ cell apoptosis was mediated by the inhibition of PI3K/Akt/mTOR signaling.

Oxidative stress plays a crucial role in the development mechanism of many diseases, including bladder cancer (Wigner et al. 2021). Although moderate levels of ROS as secondary messengers play a critical role in several intracellular signaling pathways, excessive levels of ROS can increase the oxidation of mitochondrial pores, triggering an onset of DNA damage and apoptotic signaling (Moloney and Cotter 2018; Srinivas et al. 2019). To be more specific, ROS have been shown to downregulate the cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) half-life by inducing its ubiquitin-proteasomal degradation, thus enhancing extrinsic pathway (Wilkie-Grantham et al. 2013; Perillo et al. 2020). In addition, for the majority of ROS-related anticancer drugs, apoptosis depends on the activation of the intrinsic pathway that involves mitochondrial permeability, which is increased with the cytoplasmic release of cytochrome c that forms a complex with apoptotic protease activating factor 1 (Apaf-1) and pro-caspase-9 to build the apoptosome, in turn, effector caspases (Perillo et al. 2020). In this regard, many bioactive compounds with anti-cancer activity induced apoptosis as well DNA damage in cancer cells by increasing ROS levels, which correlated with the inactivation of the PI3K/Akt/mTOR signaling pathway (Wu et al. 2019). Moreover, several previous studies suggested that the inactivation of the PI3K/Akt/mTOR signaling pathway and DNA damage were associated with excessive ROS production (Karimian et al. 2019; Cao et al. 2020). These observations imply that inhibiting PI3K/Akt/mTOR signaling through the promotion of ROS generation may be an attractive strategy for inducing cancer cell apoptosis. Therefore, we investigated whether the anticancer activity of PD was related to ROS generation and evaluated the role of ROS in the inactivation of the PI3K/Akt/mTOR signaling. Consistent with previous findings observed in breast cancer and leukemia cells (Shin et al. 2009; Yu and Kim 2012), ROS levels were markedly increased during the early stage of PD treatment. However, quenching ROS production by NAC significantly diminished PD-induced DNA damage and dephosphorylation of PI3K and Akt proteins, and blocked PD-induced apoptosis and viability reduction. These results demonstrate that PD induces DNA damage and apoptosis in EJ bladder cancer cells by triggering ROS-mediated perturbation of the PI3K/AKT/mTOR signaling pathway.

In conclusion, the current results indicated that ROS generation by PD played an important role in the induction of DNA damage and apoptosis in EJ bladder urothelial carcinoma cells and acted as an upstream signal to inhibit the activation of the PI3K/Akt/mTOR signaling pathway. However, further studies are needed to determine the role of other cellular signaling pathways that may be involved in the anti-cancer activity of PD, and determine the direct relationship with PI3K/Akt/mTOR signaling. In addition, the role of other cellular organelles contributing to ROS production by PD should be investigated, and it is necessary to verify the anticancer efficacy of PD in an *in vivo* bladder cancer model.

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