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Original article

Toyocamycin induces apoptosis via the crosstalk between reactive oxygen species and p38/ERK MAPKs signaling pathway in human prostate cancer PC-3 cells

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ABSTRACT

Background: Toyocamycin, an antibiotic agent isolated from *Streptomyces* species, has been shown to have anticancer and chemopreventive effects on various cancer cells. Until now, Toyocamycin-induced apoptosis has not been reported to be involved in the regulation between mitogen-activated protein kinases (MAPKs) and reactive oxygen species (ROS) production.
Methods: Cell viability assay, western blot, cell-cycle arrest, annexin V/propidium iodide assay, reactive oxygen species (ROS) production, mitochondrial membrane potential and intracellular Ca^{2+} flux were assayed.
Results: We investigated the apoptotic effect of Toyocamycin and the underlying molecular mechanism in prostate cancer PC-3 cells. Toyocamycin treatment resulted in reduced cell viability of PC-3 cells, but not of non-malignant RWPE-1 cells. Toyocamycin enhanced apoptosis, mitochondrial dysfunction, and ROS production in PC-3 cells. In addition, MAPK proteins were activated upon Toyocamycin treatment. The p38 and extracellular signal-regulated kinases (ERK) activities were regulated by ROS-mediated signaling pathway underlying the Toyocamycin-induced apoptosis. Pretreatment with N-acetyl-L-cysteine (NAC) recovered the Toyocamycin-induced mitochondrial dysfunction, ROS, and apoptosis. Additionally, p38 stimulated ROS production and inhibitory effects on ERK activation, while ERK inhibited the ROS production and had no effect on p38 activation.
Conclusion: ROS-mediated activation of p38/ERK partially contributes to Toyocamycin-induced apoptosis, and p38/ERK MAPKs regulate the ROS production in PC-3 cells.

Introduction

Prostate cancer is one of the most common noncutaneous malignancy in men, especially in western countries. Recently, researchers have been trying to figure out the causes of prostate cancer [1,2]. At early stages, the surgeon carries out several methods, such as radiation therapy, prostatic surgery and androgen ablation. However, in many cases, these treatment are unsuccessful, leading to relapses of androgen-independent prostate cancer [3]. Currently, the good alternative methods were not found out [3,4]. Therefore, new therapeutic targets and molecular regulations have been studied in efforts to find a cure for prostate cancer.

Many studies reported that reactive oxygen species (ROS) is associated with various cellular processes including transcription factor activation, gene expression, cell proliferation and death [5]. ROS generation is an important source for mitochondrial electron transport chain [6]. Several researches have shown that excess ROS production decreases mitochondrial membrane potential (MMP), leading to apoptotic cell death [7]. Moreover, ROS-mediated mitogen-activated protein kinases (MAPKs) activation regulates many other cellular processes, such as cell growth,

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proliferation and survival [8]. There are three conserved MAPKs, including c-jun NH_2-terminal kinases (JNK), extracellular signal-regulated kinases (ERKs), and the p38 groups [9]. In particular, p38 activation is related with chemotherapeutic agents-induced apoptotic cell death [10]. Furthermore, p38 activation is associated with oxidative stress such as ROS production [11]. In addition, recent studies have been shown that ERK activation may also lead to apoptotic cell death in response to anticancer agents or ROS production [12].

Toyocamycin is an antibiotic analogue of adenosine, isolated from the *Streptomyces* species. Toyocamycin is a potent inhibitor of RNA self-cleavage and phosphatidylinositol kinase in mammalian cells [13]. Furthermore, Toyocamycin has also been reported to inhibit kinase activities, such as protein kinase C (PKC) [14], cdc2 [15] or phosphatidyl inositol 3-kinase (PI3 K) [16]. It also inhibits ER stress-mediated X-box binding-1(XBP-1) splicing in MM cells [17]. Many adenosine analogs like Toyocamycin, Sangivamycin and MCS-C2 have been researching for anticancer therapy agents in several cancer cells [18]. However, to the best of our knowledge the signal pathway between activation of ROS-mediated MAPKs and apoptosis by Toyocamycin has not been reported until now. In this present study, our results for the first time, suggest that Toyocamycin induces apoptosis by elevating ROS production, activating MAPKs activation, and subsequently disrupting mitochondrial function, activation of caspase-3 and cleavage of PARP in human prostate cancer cells.

Materials and methods

Reagents and antibodies

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), 3,3-dihexyloxacarbocyanine ($DiOC_6$), 6-diamidino-2-phenylindole dihydrochloride (DAPI), 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and Toyocamycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin-V-FITC apoptosis Detection Kit was purchased from BD Bioscience (San Jose, CA, USA). SB203580 and U0126 were purchased from TOCRIS (Bristol, UK). The ECL Western kit was purchased from GE Healthcare (Arlington Heights, IL, USA). Antibodies for AKT, p-AKT, Bax, Bcl-2, pro-caspase 3, Cleaved-caspase-3, ERK, p-ERK, PARP, p38, p-p38 and β-Actin were purchased from Cell Signaling (Beverly, MS, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Toyocamycin was dissolved in dimethyl sulfoxide (DMSO), as a vehicle control, and stored at −20 °C until further analysis.

Cell lines and culture

Human prostate cancer PC-3 cells and human prostate normal RWPE-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The PC-3 cells were cultured in a Dulbecco's modified Eagle's minimal medium (DMEM, WelGENE Inc., Korea) supplemented with 10% fetal bovine serum (FBS, WelGENE Inc.) and 100 units/ml of penicillin and 100 μg/ml of streptomycin (WelGENE Inc.), respectively. The RWPE-1 cells were cultured in keratinocyte serum-free media (K-SFM, GIBCO, Gland Island, NY, USA) containing 2.5 μg of epidermal growth factor (EGF), 25 mg of bovine pituitary extract (BPE), and 1% penicillin-streptomycin solution. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell viability

The cell viability was measured by the MTT assay. The cells were seeded and treated with various concentrations of Toyocamycin for 24 or 48 h. After Toyocamycin treatment, MTT (1 mg/ml) was added to each well and incubated for 3 h at 37 °C. Then, the medium was removed and MTT-formazan complex was dissolved in DMSO. Absorbance was observed at 570 nm ELISA reader ($VERSA_{max}$ microplate reader, Molecular Devices, Toronto, Canada). The cell viability was determined as a relative percentage of the treated cells to one of the untreated cells by comparing optical densities.

Morphological changes

Nuclear morphological changes were measured by a fluorescence microscopy. The cells were incubated in the absence or presence of Toyocamycin for 48 h. The cells were fixed with 4% paraformaldehyde, then stained 1 mg/ml of DAPI solution for 30 min. After washing, the cells were observed under a fluorescence microscopy (AXIO Imager, ZEISS, Jena, Germany).

Annexin V/PI double staining

Apoptotic cells were measured by an annexin V-FITC staining kit. In brief, the PC-3 cells were treated with various concentrations of Toyocamycin for 48 h and then washed with PBS. The collected cells were mixed in 100 μl of 1× annexin binding buffer. After annexin V/PI double staining for 15 min, the cells were analyzed by a flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA), and the apoptotic cells were calculated using Cell Quest Pro software on Mac® OS 9 (Becton Dickinson).

Reactive oxygen species (ROS) production

ROS generation was measured by a DCFH-DA fluorescent dye. The cells were cultured in a 6-well plate at a density of 2.5×10^4/well. After treatment with various concentrations of Toyocamycin for 48 h, the cells were incubated with 10 μM of DCFH-DA at 37 °C for 30 min. The cells were then harvested, and the intensity of fluorescence was measured with flow cytometry (FACSCalibur, Becton Dickinson) and calculated using Cell Quest Pro software on Mac® OS 9 (Becton Dickinson).

Mitochondrial membrane potential (MMP, ΔY_m)

MMP was measured by dye $DiOC_6$. The cells were cultured in a 6-well plate at a density of 2.5×10^4/ml. After treatment with various concentrations of Toyocamycin for 48 h, the cells were incubated with 100 nM of $DiOC_6$ at 37 °C for 30 min and analyzed by flow cytometry (FACSCalibur, Becton Dickinson) and calculated using Cell Quest Pro software on Mac® OS 9 (Becton Dickinson).

Western blotting

The cell extracts were prepared by incubating the cells in a lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzidine, 1 mg/ml pepstatin, 8 mM sodium pyrophosphate and 20 mM β-glycerophosphate]. Forty micrograms of proteins were electrophoretically separated using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 8–15% gel and transferred to a polyvinylidenefluoride (PVDF) membrane. After blocking with a TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk, the membranes were incubated with primary and secondary antibodies. The membranes were then washed with a TBS-T buffer and visualized with ECL Western blot analysis detection reagents. The density of each band was determined with a fluorescence scanner

(LAS 3000, Fuji Film, Tokyo, Japan) and analyzed with Multi Gauge V3.0 software (Fuji Film).

Statistical analysis

All experiments were repeated at least three times. Unless otherwise stated, data are expressed as the mean ± SD. Analysis of variance was used to compare the experimental groups to the control values, unless comparisons between multiple groups were performed by Tukey's multiple comparison test using GraphPad InStat V3.05. Results were statistically significant at $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the untreated group.

Results

Toyocamycin reduces cell viability and induces apoptosis in human prostate cancer PC-3 cells

To investigate the anticancer effect of Toyocamycin in human androgen-independent PC-3 prostate cancer cells and non-malignant RWPE-1 prostate epithelial cells, the cells were treated with various concentrations (0–100 nM) of Toyocamycin for indicated times. Toyocamycin significantly decreased the cell viability of PC-3 cell lines in a dose- and time-dependent manner (Fig. 1A). By comparison, the RWPE-1 cells were less sensitive to Toyocamycin than the malignant cancer PC-3 cells (Fig. 1B). In addition, nuclear condensation and fragmentation were characterized by DAPI staining (red arrows) (Fig. 1C), suggesting that morphological changes by Toyocamycin may indicate apoptotic cell death in the PC-3 cells. Next, we confirmed whether the cell growth inhibition by Toyocamycin is associated with the induction of apoptosis in the PC-3 cells. As shown in Fig. 1D, the apoptotic cells were increased from 2 to 62% in a dose-dependent manner at 0–100 nM of Toyocamycin in the PC-3 cells. Furthermore, we evaluated the expression of apoptosis-related proteins by a Western blot analysis. As shown in Fig. 1E, Toyocamycin did not affect Bax expression but a decreasing effect on Bcl-2 expression. Thus, the ratio of Bax/Bcl-2 was increased in a dose-dependent manner, indicating Toyocamycin-induced apoptosis [19]. In addition, Toyocamycin induced caspase-3 activation and PARP cleavage in a dose-dependent manner. Next, we calculated the levels of MMP by Toyocamycin treatment in PC-3 cells. The hyperpolarization of MMP significantly increased up to 100 nM of Toyocamycin (Fig. 1F). These results suggested that Toyocamycin-induced apoptosis is associated with the alteration of Bcl-2 family expression and mitochondrial dysfunction in the PC-3 cells.

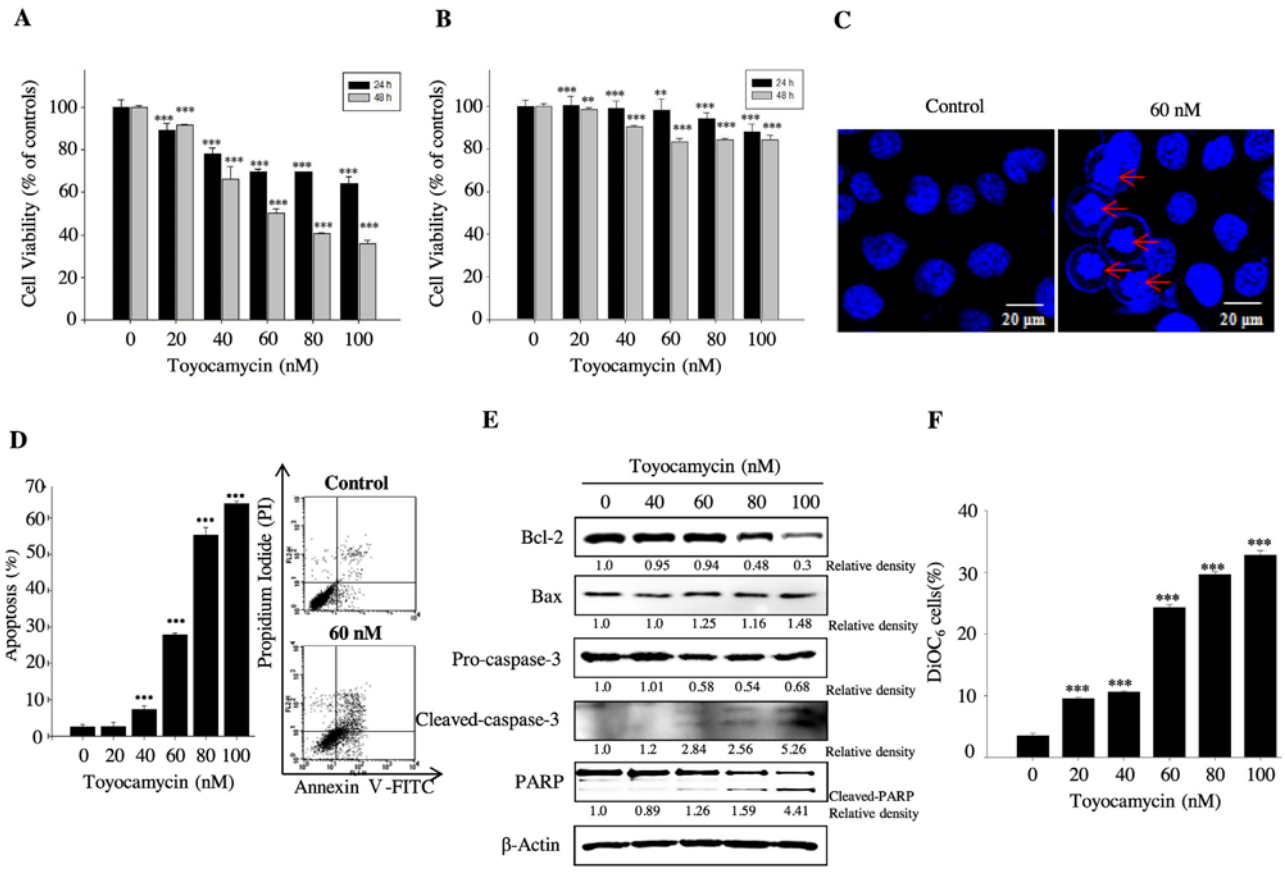

Fig. 1. Toyocamycin-induced apoptosis in human prostate cancer PC-3 cells. Cell viability (A) PC-3, and (B) RWPE-1 cells. The cells were treated with Toyocamycin (0–100 nM) for 24 or 48 h. Cell viability was determined by MTT assay. (C) Morphological changes. The cells were incubated with 60 nM of Toyocamycin for 48 h. The cell morphology was observed by using phase-contrast microscopy (×400). Nuclear morphological changes were measured by using a fluorescence microscopy after DAPI staining at room temperature for 10 min. Nuclear condensation was marked with red arrows (×200). (D) Apoptosis induction. The cells were treated with various concentration of Toyocamycin for 48 h (E) Bcl-2, Bax, Pro-, cleaved caspase-3 and PARP levels. The cells were treated with 60 nM of Toyocamycin at different time points (12, 24, 36 and 48 h). Equal amounts (40 μg) of protein lysates were used for Western blotting. *lower panel* shows relative intensity of proteins. (F) Mitochondrial membrane potential (MMP). The cells were incubated with 100 nM of $DiOC_6$ at 37 °C for 30 min and analyzed by a flow cytometry. Data are presented as the mean ± SD ($n = 3$ in each group); $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* untreated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ROS mediates toyocamycin-induced apoptosis via *mitochondrial pathway*

Recent studies have shown that anticancer drugs induce apoptosis, in part, by triggering ROS generation [5]. However, significant ROS production by Toyocamycin has not been reported in cancer cells, thus far. To investigate whether ROS are generated from the Toyocamycin-treated cells, we determined the state of ROS by examining DCFH-DA fluorescence intensity. As a result, Toyocamycin significantly triggered ROS production in PC-3 cells (Fig. 2A). On the other hand, the pretreatment of NAC significantly reduced ROS production induced by Toyocamycin (Fig. 2B). Many studies have reported that ROS production is highly related to mitochondria-mediated apoptosis [20]. Therefore, we confirmed whether Toyocamycin-induced apoptosis is associated with ROS production in PC-3 cells. Pretreatment with NAC recovered the hyperpolarization of MMP and apoptosis induced by Toyocamycin (Fig. 2C, D). Furthermore, the addition of NAC significantly rescued the expression of apoptosis-related proteins, such as pro-caspase-3, cleaved caspase-3 and PARP (Fig. 2E). These findings suggest that an increase of ROS generation may take part in mitochondria-mediated apoptosis induced by Toyocamycin in PC-3 cells.

Toyocamycin regulates the crosstalk of ROS and p38/ERK activation

Several reports have shown that PI3K/AKT and MAPKs signaling pathways are involved in the pathogenesis of cancer and induction of apoptosis [8]. To investigate the roles of PI3K/AKT and MAPKs pathways in Toyocamycin-induced apoptosis, we examined the expression levels of AKT, ERK, p38 and JNK phosphorylation by a Western blot analysis. As shown in Fig. 3A, Toyocamycin significantly reduced the phosphorylation of AKT and increased the phosphorylation of ERK and p38, but it did not detect the phosphorylation of JNK. Thus, we confirmed the relationship between MAPKs and ROS in PC-3 cells. Previous studies have identified that ROS can induce or mediate the activation of MAPKs pathways [21]. We showed that pretreatment with NAC reduced both ERK and p38 activities (Fig. 3B) and recovered the activation of AKT (data not shown). We further confirmed whether ERK and p38 activations play a role in ROS production. Pretreatment with SB203080 (p38 inhibitor) partially recovered the production of Toyocamycin-induced ROS (Fig. 3C). In contrast, pretreatment with U0126 (ERK inhibitor) enhanced ROS production (Fig. 3D). These results indicated that the inhibition of ROS production suppressed the activities of ERK and p38. Moreover, the inhibition of p38 and ERK activities differentially regulated ROS generation, suggesting that the crosstalk between ROS production and MAPKs activation may be associated with the regulation of Toyocamycin-induced apoptosis in PC-3 cells. These results indicated that ROS have the crosstalk with p38/ERK MAPKs activation for its homeostasis in PC-3 cells.

Toyocamycin regulates ROS-mediated apoptosis by inhibition of p38 on ERK activation

To investigate the regulation of ROS by MAPKs inhibitor on apoptosis, we pretreated the p38 inhibitor or ERK inhibitor with Toyocamycin in PC-3 cells. The p38 inhibitor, SB203582, not only significantly prevented the induction of apoptosis (Fig. 4A), but also recovered apoptosis-related protein expressions (Fig. 4B). However, the ERK inhibitor, U0216, synergistically enhanced apoptotic cell death (Fig. 4C), resulting from increased active-form of apoptosis-related proteins (Fig. 4D). The phenomena were consistent with ROS production generated by MAPKs inhibitors after treatment of Toyocamycin, as shown in Fig. 3C and D. Moreover these results suggest that p38 activation may be related

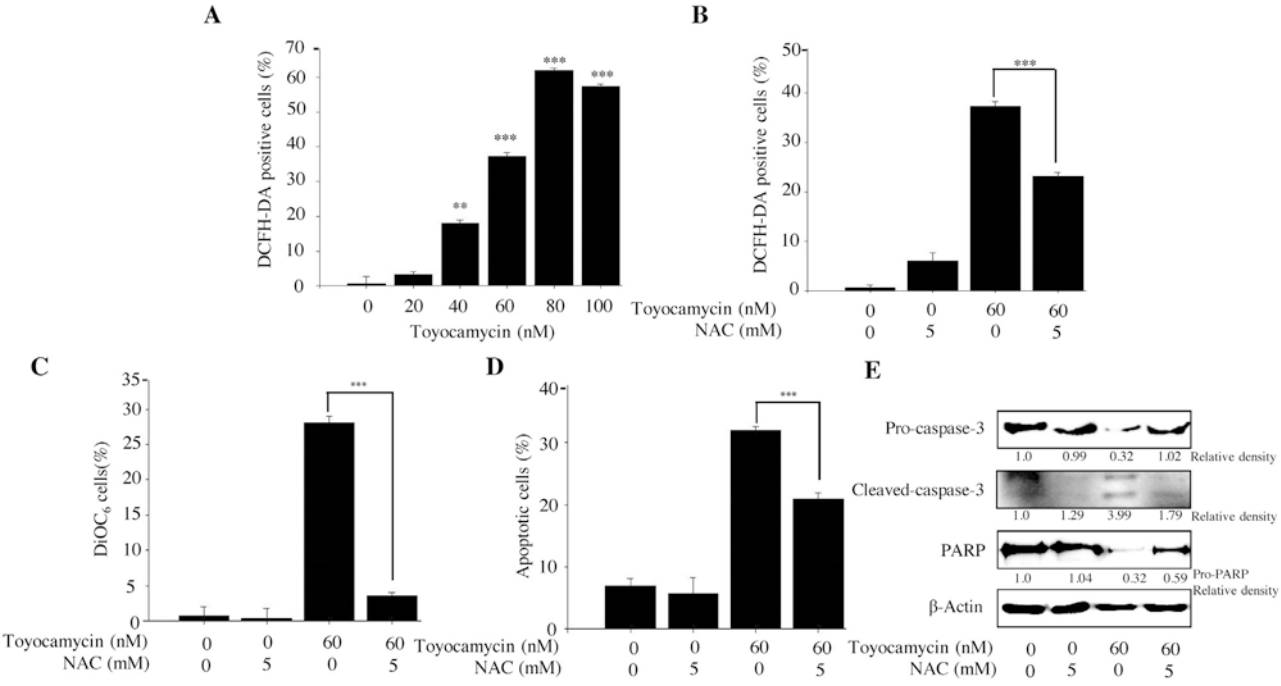

Fig. 2. Toyocamycin induces apoptosis *via* reactive oxygen species-mediated mitochondrial pathway in human prostate cancer PC-3 cells. **(A)** Intracellular ROS levels. The cells were treated with various concentrations of Toyocamycin for 48 h. The ROS generation was detected with a fluorescence dye of DCFH-DA by a flow cytometry. (B) The inhibition of ROS generation by NAC. The cells were treated with 60 nM of Toyocamycin for 48 h with or without pretreatment using 5 mM NAC for 1 h (C) Recovery of MMP dysfunction by NAC. MMP was detected with a fluorescence dye of $DiOC_6$ by flow cytometry. (D) Recovery of apoptosis by NAC. Apoptosis was detected with annexin-V/propidium iodide (PI) staining by flow cytometry. (E) Regulation of apoptosis-related proteins expressed by NAC. Equal amounts (40 μg) of protein lysates were used for Western blotting. *lower panel* shows relative intensity of proteins. Data are presented as the mean ± SD ($n = 3$ in each group) ; $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* untreated group.

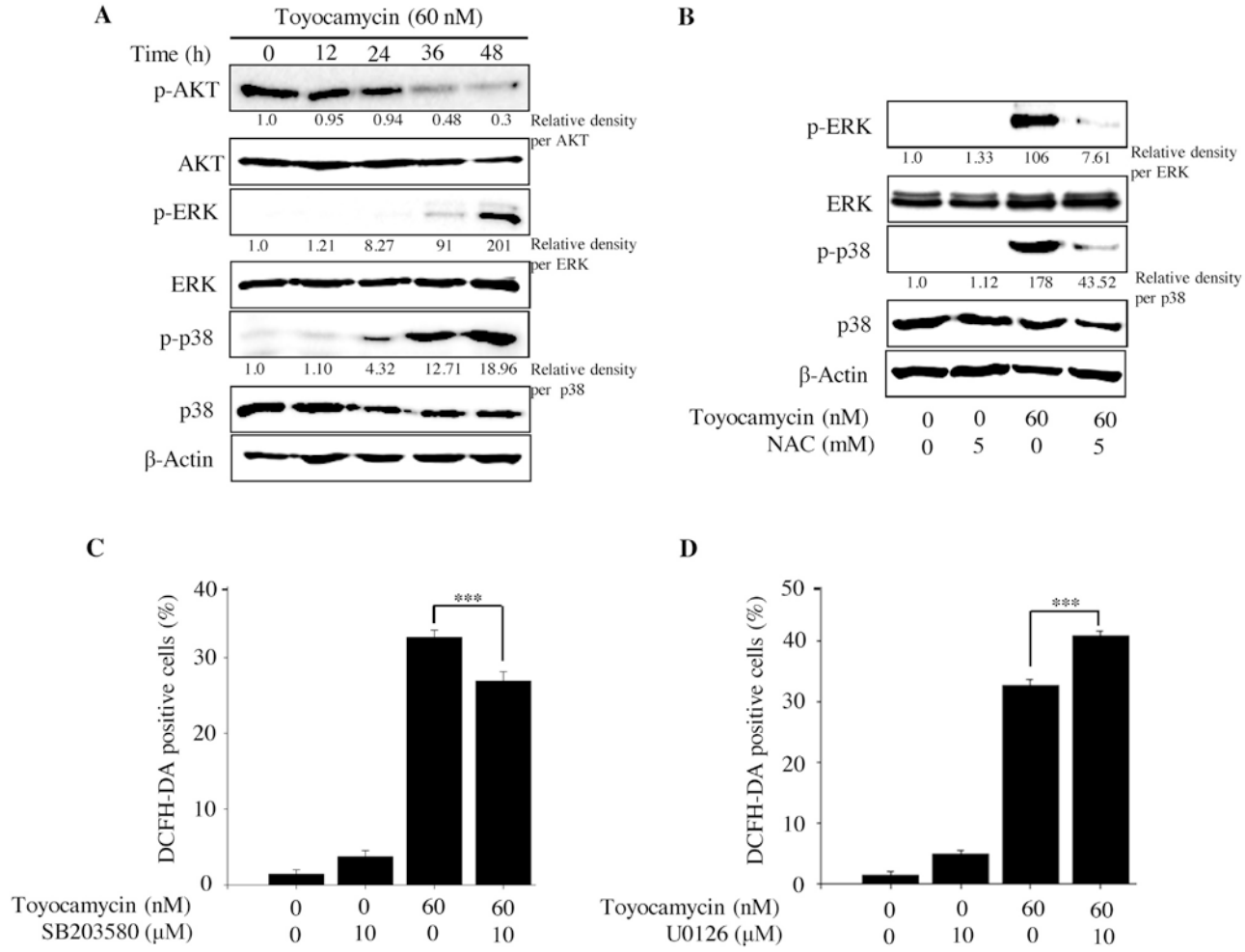

Fig. 3. Toyocamycin induces apoptosis through p38/ERK activation in human prostate cancer PC-3 cells. (A) Phosphorylation of AKT, ERK and p38. The cells were treated with 60 nM of Toyocamycin at different time points (12, 24, 36 and 48 h). Equal amounts (40 μg) of protein lysates were used for Western blotting. *lower panel* shows relative intensity of proteins. (B) The effects of p38 and ERK protein activity by NAC. The cells were treated with 60 nM of Toyocamycin for 48 h with or without pretreatment using 5 mM NAC for 1 h, respectively. Equal amounts (40 μg) of protein lysates were used for Western blotting. *lower panel* shows relative intensity of proteins. (C, D) Regulation of ROS production by SB203580 (p38 inhibitor) and U0126 (ERK inhibitor). The cells were treated with 60 nM of Toyocamycin for 48 h with or without pretreatment with 10 μM SB203580 and 10 μM U0126 for 1 h, respectively. The ROS production was detected with a fluorescence dye of DCFH-DA by a flow cytometry. Data are presented as the mean ± SD ($n = 3$ in each group); $^{***}p < 0.001$ *vs.* untreated group.

to a positive regulation and that ERK may negatively impact apoptosis in PC-3 cells. We also checked the crosstalk between p38 and ERK. Several reports have shown that p38 was regulated by the expression of ERK [22]. As expected, p38 inhibitor promoted ERK activation, which indicated that p38 negatively regulated ERK (Fig. 4E). In contrast, the ERK inhibitor had no impact on the expression of active p38 (Fig. 4F). As summarized, p38 inhibited the activation of ERK, while ERK did not have any effect on p38 activation. Taken together, Toyocamycin-induced apoptosis is regulated by apoptotic molecule p38 and survival molecule ERK. Additionally, Toyocamycin-induced apoptosis is regulated by the crosstalk between ROS and p38/ERK MAPKs in PC-3 cells.

Discussion

Toyocamycin is an adenosine analog and biosynthetic product of the bacterium *Streptomyces* species [13]. Recently, many adenosine analogs, such as Sangivamycin and MCS-C2, potently inhibited cancer cell cycle and induced apoptosis [18,23]. Recent results showed that Toyocamycin also inhibited rRNA processing, causing a decrease in cell viability [19,24]. Our data showed that, in case of Toyocamycin, androgen-independent PC-3 human prostate cancer cells were more susceptible to cell viability than non-malignant RWPE-1 prostate epithelial cells. Such results indicate that Toyocamycin selectively kills cancer cells. Previously, it has been reported that excessive ROS production induces apoptosis through several mechanisms such as mitochondrial damage and consequent release of cytochrome c [5]. Moreover, the ratio of Bax/Bcl-2 is very important for the regulation of mitochondria-mediated apoptosis [25]. Our results provided clear evidence of Toyocamycin-induced ROS production, mitochondrial membrane hyperpolarization and caspase-3 activation and these effects were recovered by NAC pretreatment, which is a ROS scavenger.

Several reports suggested that MAPKs are involved in the signaling pathways in response to various extracellular stimuli, resulting in apoptosis [26]. It was demonstrated that p38 activation is mediated in apoptosis by diverse anticancer drugs [27,28]. Several papers have shown that p38 directly regulates the pro- and/or anti-apoptotic proteins or negatively modulates the PI3 K/AKT and ERKs survival pathways [29,30]. Moreover, p38 can act as a tumor suppressor [31].

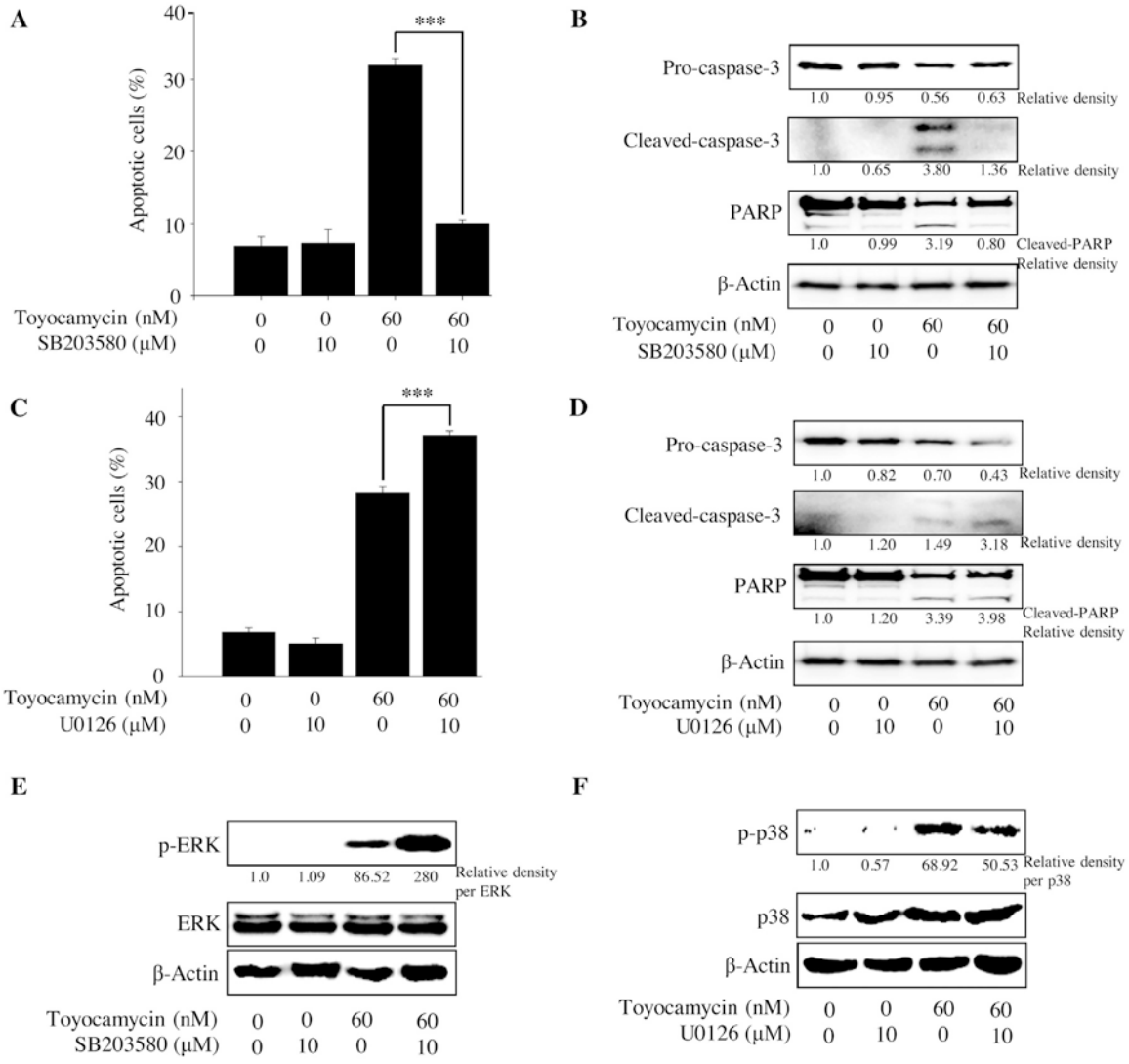

Fig. 4. Toyocamycin regulates apoptosis by crosstalk between ROS and p38/ERK MAPKs in human prostate cancer PC-3 cells. (A) Recovery of apoptosis by SB203580 (p38 inhibitor). (B) Effects of apoptosis-related protein by SB203580. (C) Regulation of apoptosis by U0126 (ERK inhibitor). (D) Effects of apoptosis-related protein by U0126. The cells were treated with 60 nM of Toyocamycin for 48 h with or without pretreatment with 10 μM SB203580 and 10 μM U0126 for 1 h, respectively. (E) Effects of ERK protein activity by SB203580. (F) Effects of p38 protein activity by U0126. The cells were treated with 60 nM of Toyocamycin with or without each inhibitor for 48 h. Equal amounts (40 μg) of protein lysates were used for Western blotting. *lower panel* shows relative intensity of proteins. Data are presented as the mean ± SD ($n = 3$ in each group); $^{***}p < 0.001$ *vs.* untreated group.

On the basis of recent reports regarding the relationship between ROS and MAPKs in apoptosis, it has been well established that p38 activation contributes to a positive feedback loop, such as ROS/p38 MAPK cascade for anticancer drugs-induced apoptosis [32]. Toyocamycin repressed AKT and JNK activity, but activated ERK and p38. Our data confirmed that NAC pretreatment reduced the Toyocamycin-induced activation of p38 and ERK. Furthermore, we demonstrated that SB203580 (p38 inhibitor) partially reduced ROS production, but U0126 (ERK inhibitor) enhanced it. Consistent with previous results, our data showed that Toyocamycin significantly increased p38 phosphorylation in a time-dependent manner. Toyocamycin-induced apoptosis was recovered by SB203580. In contrast to p38, Toyocamycin-induced apoptosis was more increased by U0126, suggesting that the activation of p38/ERK MAPKs is one of the important pathways to Toyocamycin-induced apoptosis. Previous studies have shown that sangivamycin, an adenosine analog, induces apoptosis by inhibiting ERK phosphorylation in primary effusion lymphoma cells [33]. Compared with these, our result showed that the effect of Toyocamycin has a critical role in ERK-activated cytoprotection, suggesting that the impact of ERK signaling may depend on the type and status of tumor and stimuli. That is to say, Toyocamycin-induced apoptosis was regulated by MAPKs of apoptotic molecule p38 and survival molecule ERK. Taken together, these results demonstrate that both production of ROS and regulation of MAPKs are involved in Toyocamycin-induced apoptosis. Toyocamycin promotes the production of ROS, which induces activation of ERK and p38, suggesting that ROS-mediated p38/ERK MAPKs pathway plays a dual role in cancer treatment. Moreover, additional results from this present study show that Toyocamycin-induced apoptosis may be regulated by the crosstalk between ROS and p38/ERK MAPKs, which were confirmed by pharmacologic approaches, including each inhibitor (Fig. 5). Interestingly, ERK signaling acts as a cell survival role in Toyocamycin-induced apoptosis, whereas p38 promotes cell death cascade. In conclusion, our study

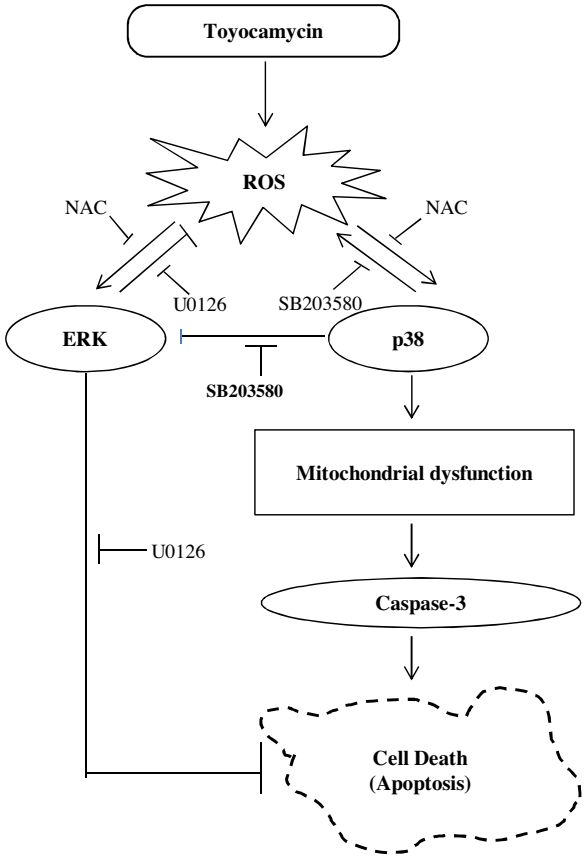

Fig. 5. Schematic drawing representing possible mechanisms underlying Toyocamycin-induced apoptosis in human prostate cancer PC-3 cells. Toyocamycin induces apoptosis through the regulation of p38/ERK MAPK and ROS in PC-3 cells.

provided new insights into the role of ROS- and MAPKs-activated signals in Toyocamycin-induced apoptosis in PC-3 cells.

Conflict of interest

There are no conflicts of interest.

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Not applicable.

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