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β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation

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ABSTRACT

Both PI3K/AKT/mTOR/S6K1 and mitogen activated protein kinase (MAPK) signaling cascades play an important role in cell proliferation, survival, angiogenesis, and metastasis of tumor cells. In the present report, we investigated the effects of β-caryophyllene oxide (CPO), a sesquiterpene isolated from essential oils of medicinal plants such as guava (*Psidium guajava*), oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum* spp.) clove (Eugenia caryophyllata), and black pepper (*Piper nigrum* L.) on the PI3K/AKT/mTOR/S6K1 and MAPK activation pathways in human prostate and breast cancer cells. We found that CPO not only inhibited the constitutive activation of PI3K/AKT/mTOR/S6K1 signaling cascade; but also caused the activation of ERK, JNK, and p38 MAPK in tumor cells. CPO induced increased reactive oxygen species (ROS) generation from mitochondria, which is associated with the induction of apoptosis as characterized by positive Annexin V binding and TUNEL staining, loss of mitochondrial membrane potential, release of cytochrome c, activation of caspase-3, and cleavage of PARP. Inhibition of ROS generation by N-acetylcysteine (NAC) significantly prevented CPO-induced apoptosis. Subsequently, CPO also down-regulated the expression of various downstream gene products that mediate cell proliferation (cyclin D1), survival (bcl-2, bcl-xL, survivin, IAP-1, and IAP-2), metastasis (COX-2), angiogenesis (VEGF), and increased the expression of p53 and p21. Interestingly, we also observed that CPO can significantly potentiate the apoptotic effects of various pharmacological PI3K/AKT inhibitors when employed in combination in tumor cells. Overall, these findings suggest that CPO can interfere with multiple signaling cascades involved in tumorigenesis and used as a potential therapeutic candidate for both the prevention and treatment of cancer.

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1. Introduction

Plants have long been used in the treatment of cancers such as prostate and breast cancer, but neither their active constituents nor the molecular targets have yet been fully understood. One potential agent is β-caryophyllene oxide (CPO), which is isolated from the essential oils of various medicinal and edible plants such as guava (*Psidium guajava*), oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum*

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spp.) clove (Eugenia caryophyllata), and black pepper (*Piper nigrum* L.) [1,2]. In general, essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants by hydro- or steam-distillation [3]. The main constituents of essential oils, for example, sesquiterpenes like CPO exhibit a wide variety of pharmacological effects, demonstrating antibacterial, antifungal, immunomodulatory, anti-inflammatory, antirheumatic, antioxidant and anticancer properties [3]. Interestingly, the leaves of the guava tree which is one of the major sources of CPO are recommended for a variety of inflammatory conditions including gastrointeritis, uterine hemorrhage, chronic diarrhea, and swollen legs (http://www.rain-tree.com/). Also, the young leaves and shoots are used for the treatment of dysentery, inflammation of the kidneys [4], and diarrhea [5]. For example in one report, CPO was reported to exhibit significant anti-inflammatory effects in carrageenan-induced paw edema [6] and cotton pellet-induced granuloma models [7]. Another study exhibited the anti-inflammatory, antipyretic activity and anti-arthritic activity of CPO in rats [8]. CPO has also been found to induce the activation of the detoxifying enzyme glutathione S-transferase in the mouse liver and small intestine [9]. CPO has been reported to act as a potent anti-carcinogenic agent [9], exhibits cytotoxic effects against several tumor cell lines [10,11] and also demonstrates anti-platelet aggregation activity *in vitro* [12].

How CPO mediates its diverse pharmacological effects is not completely understood. Because constitutive activation of AKT/mTOR/S6K1 signaling cascades is closely linked with tumorigenesis, metastasis, and angiogenesis in human prostate and breast cancers [13–16], we postulated that CPO mediate its effects, in part, through the inhibition of the AKT/mTOR/S6K1 pathways. The phosphatidylinositol 3-kinase (PI3K)/AKT signal transduction pathway plays a pivotal role in cell survival and the enhanced protection of human prostate and breast cancer cells from apoptosis during tumorigenesis [17–19]. Besides the AKT/mTOR/S6K1 pathways, mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases that play an important role in the regulation of many cellular processes including cell growth and proliferation, differentiation, and apoptosis. MAPKs consist of growth factor-regulated extracellular signal-related kinases (ERKs), and the stress-activated MAPKs, c-jun NH_2-terminal kinases (JNKs) and p38 MAPKs [20]. Recent reports indicate that reactive oxygen species (ROS) generation, by which most anti-cancer agents act, is in part responsible for the induction of apoptosis in a wide variety of tumor cells, including prostate [21] and breast cancer [22]. Prior studies have also indicated that ROS can induce or mediate the activation of the MAPK pathways [23,24] and the activation of ERK, JNK, and p38 MAPK signaling proteins are involved in growth arrest and apoptosis via reactive oxygen species (ROS) generation [25–29].

In this study, we investigated whether CPO exerts its anti-cancer effects through the modulation of the PI3K/AKT/mTOR/S6K1 and MAPK signaling. Our results demonstrate for the first time that CPO can suppress constitutive PI3K/AKT/mTOR/S6K1 signaling cascades and induce ROS-mediated MAPK activation. This led to the inhibition of cell proliferation and the down-regulation of various gene products that prevent apoptosis, promote inflammation, angiogenesis, and metastasis in cancer cells.

2. Materials and methods

2.1. Reagents

β-Caryophyllene oxide (CPO), with a purity greater than 95%, was kindly provided by Dr. Somi Cho Kim (Jeju National University, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), N-acetyl-L-cystein (NAC), fluorescein diacetate (FDA) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). Rhodamine 123, MitoTracker and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR). Anti-p-PI3K (Tyr458), anti-PI3K, anti-p-AKT (Ser473), anti-AKT, anti-p-mTOR (Ser2448), anti-mTOR, anti-p-S6K1 (Thr421/Ser424), anti-S6K1, anti-p-ERK (Thr202/Tyr204), anti-ERK, anti-p-JNK (Thr183/Tyr185), anti-JNK, anti-p-p38 (Thr180/Tyr182), anti-p38, anti-procaspase-3, anti-cleaved caspase-3, anti-cytochrome c, anti-p-4E-BP1 (Ser65) and anti-4E-BP1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2, anti-Bcl-xL, anti-Survivin, anti-IAP-1, anti-IAP-2, anti-Cyclin D_1, anti-COX-2, anti-VEGF, anti-PARP, anti-p21, anti-p53, anti-β-actin antibodies, and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V was from BD Biosciences (Palo Alto, CA). TUNEL assay kit was from Roche Diagnostics GmbH (Germany). Wortmannin and AKT inhibitor IV were from Calbiochem (Nottingham, UK). Rapamycin and NVP-BEZ235 were from Cayman Chemical Company (Ann Arbor, MI).

2.2. Cell culture

PC-3 and MCF-7 cells were grown in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed one day after seeding the cells.

2.3. MTT assay

Cell viability was measured by an MTT assay to detect NADH-dependent dehydrogenase activity. Fifty microliters of MTT solution (5 mg/ml) in 1× phosphate-buffered saline (PBS) was directly added to the cells, which were then incubated for 4 h to allow MTT to metabolize to formazan. Absorbance was measured with an automated spectrophotometric plate reader at a wavelength of 570 nm. Cell viability was normalized as relative percentages in comparison with untreated controls.

2.4. Western blot analysis

After PC-3 and MCF-7 cells were treated with the indicated concentrations of CPO, the cells were lysed and the

total protein concentration was determined by Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of lysates resolved on sodium dodecyl-polyacrylamide gel electrophoresis (SDS–PAGE) were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), and the membrane was blocked with 1× TBS containing 0.1% Tween 20 and 5% skim milk or 2% BSA for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with the respective primary antibodies. The membranes were washed thrice and incubated with diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. After three washes, the membranes were detected using the enhanced chemiluminescence (ECL) kit (Millipore, Bedford, MA).

2.5. Cell cycle analysis

PC-3 cells were seeded onto 6-well plates at a density of 1×10^6 cells per well and incubated for one day. After treatment with 50 μM of CPO for 24 h, the cells were collected and washed with 1× PBS. Cell pellets were fixed in 70% cold ethanol overnight at −20 °C. The fixed cells were resuspended in 1× PBS containing 1 mg/ml RNase A, incubated for 1 h at 37 °C, and the cells were stained by adding 50 μg/ml PI for 30 min at room temperature in the dark. The DNA contents of the stained cells were analyzed using CellQuest Software with a FACS Vantage SE (Becton Dickinson, Heidelberg, Germany) flow cytometry (Becton Dickinson).

2.6. Annexin V analysis

1×10^6 cells were treated with CPO for 24 h, fixed with 4% paraformaldehyde, and stained by Annexin V conjugated to FITC or with 1 μg/ml DAPI solution. The cells were washed and observed accordingly with a flow cytometry (Becton Dickinson) or a fluorescence microscope.

2.7. TUNEL assay

Individual apoptotic cell death was observed using a TUNEL assay kit. For the flow cytometer based assay, PC-3 cells were seeded onto 6-well plates at a density of 1×10^6 cells per well, incubated for one day, and then treated with CPO for 24 h. The cells were collected and washed with 1× PBS. Cell pellets were fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were permeabilized by 0.2% Triton X-100 in 1× PBS for 15 min, washed, resuspended in 1× PBS containing TUNEL reaction solution, and incubated for 1 h at 37 °C in the dark. For the fluorescence microscope assay, the cells were seeded onto poly-L-lysine-coated slides, fixed with 4% paraformaldehyde for 30 min at room temperature, and washed three times with 1× PBS. 15 min after permeabilization with 0.2% Triton X-100, the cells were washed three times with 1× PBS and added to the TUNEL reaction solution. The cells were covered with aluminum foil and incubated for 1 h at 37 °C in a humidified CO_2 incubator. The cells were then stained with 1 μg/ml DAPI solution for 5 min at room temperature in the dark.

2.8. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

Rhodamine123 was used as the mitochondrial membrane potential sensitive dye. 24 h after treatment with CPO, the cells were washed with 1× PBS and incubated with 10 μM rhodamine123 for 30 min at room temperature in the dark. The cells were washed with 1× PBS and fixed with 4% paraformaldehyde. Next, the cells were stained with 1 μg/ml DAPI solution, mounted, and analyzed with a flow cytometry (Becton Dickinson) or a fluorescence microscope.

2.9. Measurement of intracellular reactive oxygen species

PC-3 cells were seeded onto 6-well plates at a density of 1×10^6 cells/well and incubated for 24 h. Afterwards, the cells were treated with 50 μM CPO and 5 mM NAC for 24 h. Then, the cells were washed twice, stained with 50 μM 2,7-dichlorofluorescin-diacetate (DCFH-DA) (Molecular Probes) for 5 min and washed twice. The esterified form of DCFH-DA can permeate cell membranes and thereafter deacetylated by intracellular esterases. The resulting compound, dichlorofluorescin (DCFH), reacts with hydrogen peroxide or ROS to form the fluorescent compound, dichlorofluorescin (DCF). The amount of intracellular fluorescent DCF was measured using flow cytometry (Becton Dickinson).

2.10. Live/dead assay

After the PC-3 cells (1×10^6 cells/well) were treated with 50 μM CPO and 5 mM NAC for 24 h, the cells were washed with 1× PBS, and stained with FDA and PI for 5 min. FDA is a non-fluorescent cell permeable dye producing a green in live cells; PI enters cells with damaged membranes and binds to nucleic acids producing a bright red fluorescence in dead cells. Data were analyzed using a confocal microscopy.

2.11. Immunocytochemistry

PC-3 cells were with CPO for 24 h, washed with 1× PBS, and then fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Next, the cells were washed three times in 1× PBS, were permeabilized with 0.2% Triton X-100 in PBS for 20 min, washed three times in PBS, and then blocked with 5% BSA in 1× PBS for 1 h at room temperature. After then, the cells were incubated with anti-cleaved caspase-3 or anti-Cytochrome c antibodies for overnight at 4 °C, washed three times, and incubated with Alexa-488 and Alexa-543-conjugated secondary antibodies for 2 h at room temperature. The cells were washed three times, mounted on glass slides, and viewed on a confocal LSM 510 Laser Scanning microscope (Zeiss, Göttingen, Germany).

2.12. Statistical analysis

All numeric values are represented as the mean ± SD. Statistical significance of the data compared with the untreated control was determined using the Student unpaired *t*-test. Significance was set at $P < 0.05$.

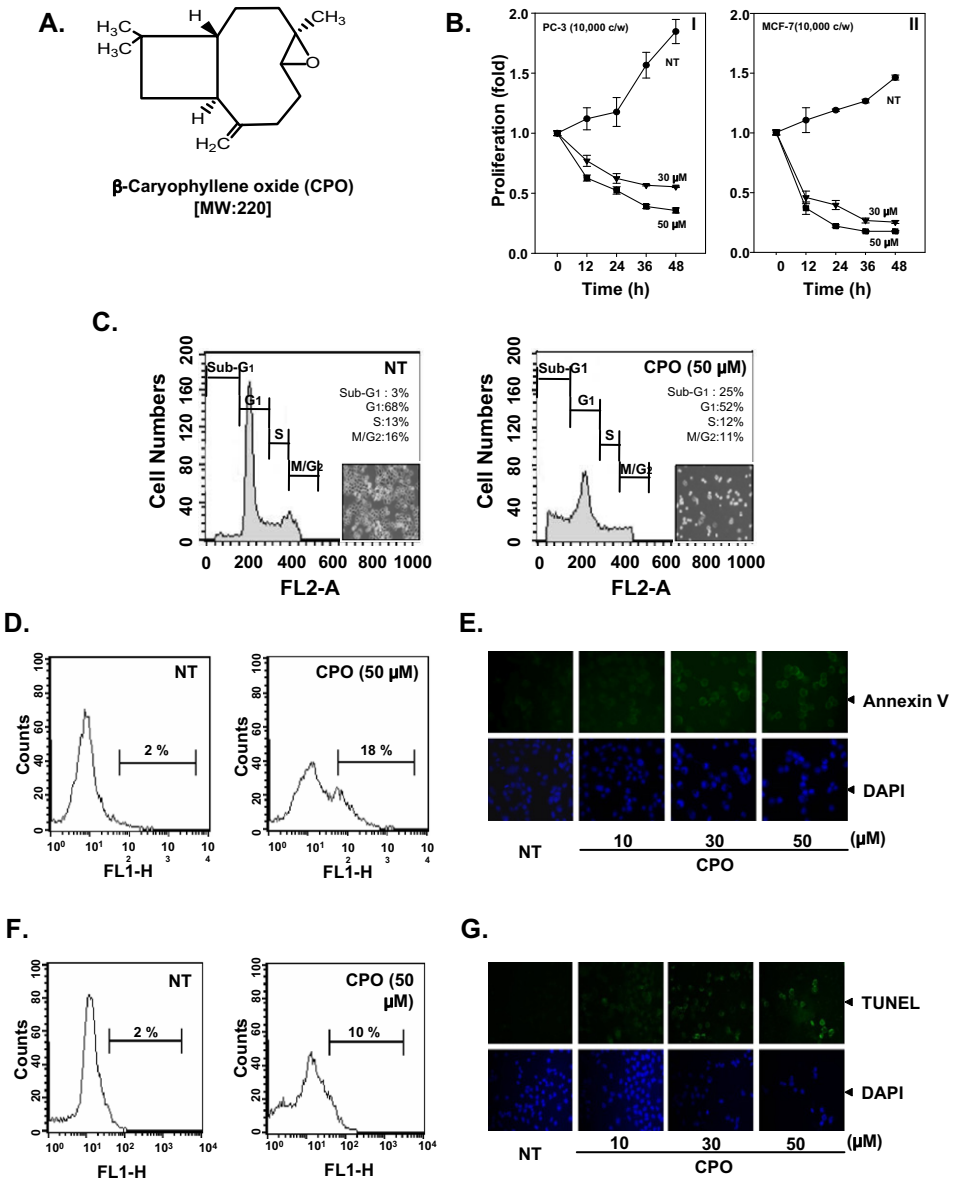

Fig. 1. CPO suppressed cell proliferation and induced apoptosis in tumor cells. (A) The chemical structure of β-caryophyllene oxide, CPO. (B) After PC-3 (I) and MCF-7 (II) cells (1×10^4 cells/well) were seeded onto 96-well plates, they were left non-treated (NT, ●) or treated with CPO at 30 μM (▼) and 50 μM (■) concentrations for the indicated time intervals. The cell proliferation was measured using the MTT assay. (C) After 1×10^6 cells were seeded onto 6-well plates, they were treated with 50 μM of CPO for 24 h. Then, the cells were fixed and analyzed using a flow cytometer, or observed using a microscope (*inset*). PC-3 cells were treated with indicated concentrations of CPO for 24 h and the cells were incubated with an FITC-conjugated Annexin V antibody and then analyzed by a flow cytometry (D) and under a fluorescence microscope (E) as described in Section 2. After treatment of CPO for 24 h, the cells were fixed and incubated using TUNEL reaction solution and then analyzed by a flow cytometry (F) and under a fluorescence microscope (G) as described in experimental procedures.

3. Results

3.1. CPO suppresses cell proliferation in human prostate cancer PC-3 and breast cancer MCF-7 cells

We previously have fractioned extracts of guava leaves in MeOH, BuOH, $CHCl_3$, Hexane, H_2O, and EtOAc. Among them, the guava hexane fraction (GHF) was found to exhibit most potent cytotoxic effects in PC-3 cells (data not shown). To specifically examine the anti-tumor activity of a single component CPO obtained from GHF on PC-3 and MCF-7 cells (Fig. 1A), the cells were treated with 30 or 50 μM concentrations of CPO for indicated time intervals, and then cell viability was analyzed using a MTT assay. As shown in Fig. 1B, CPO significantly suppressed cell proliferation in these tumor cells in a time dependent manner. Also, PI staining revealed the peaks that represent the increased percentage in apoptotic cell population (sub-G_1): from 3% (*left*) to 25% (*right*) on treatment, moreover CPO also induced

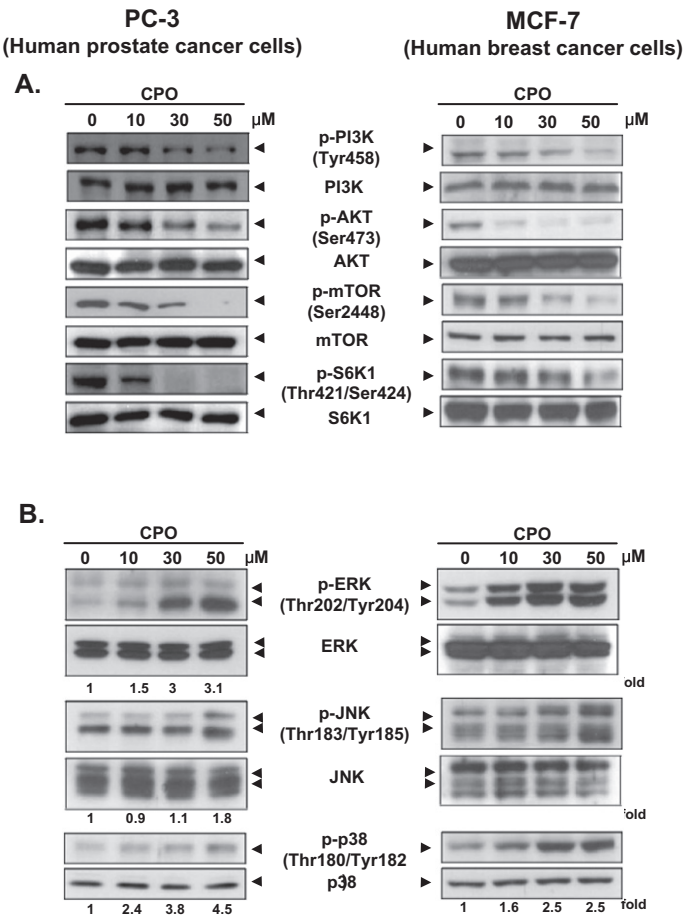

Fig. 2. CPO suppressed PI3K/AKT/mTOR/S6K1 and MAPKs signaling pathways in human prostate and breast cancer cells. (A) PC-3 and MCF-7 cells were treated with various indicated concentrations of CPO for 6 h. Then equal amounts of lysates were analyzed by Western blot analysis using antibodies against p-PI3K (Tyr458), PI3K, p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-S6K1 (Thr 421/Ser424), and S6K1. (B) Cells were treated with various indicated concentrations of CPO for 6 h. Then equal amounts of lysates were analyzed by Western blot analysis using antibodies against p-ERK (Thr202/Tyr204), ERK, p-JNK (Thr183/Tyr185), JNK, p-p38 (Thr180/Tyr182), and p38. The results shown here are representative of three independent experiments.

morphological changes characteristic of apoptosis (*inset*) (Fig. 1C). These data suggest that CPO treatment leads to the suppression of cell proliferation and induces apoptosis as evident by the increased accumulation of the cells in the sub-G_1 phase of the cell cycle.

3.2. CPO induces early and late apoptosis

To further demonstrate the anti-cancer effects of CPO, we examined early apoptosis using the Annexin V antibody. The Annexin V positive cells (regarded as early apoptotic cells) were increased as compared with the non-treated cells as observed by flow cytometric analysis (Fig. 1D) or under the fluorescence microscope (Fig. 1E). When we further examined for late apoptosis by analyzing DNA strand breaks using the TUNEL assay, apoptotic cells were also significantly increased on CPO treatment as observed by flow cytometric analysis (Fig. 1F) or fluorescence microscopy (Fig. 1G).

3.3. CPO inhibits PI3K/AKT/mTOR/S6K1 signaling cascades

PI3K/AKT/mTOR/S6K1 are the major anti-apoptotic pathways that confer the survival advantage and resistance of cancer cells against various chemotherapeutic agents [14]. We first investigated whether CPO down-regulates constitutive PI3K/AKT/mTOR/S6K1 activation in tumor cells. As shown in Fig. 2A, constitutive activation of both PI3K, as well as the serine/threonine protein kinase AKT was suppressed by CPO in a concentration-dependent manner. In addition, constitutive mTOR and S6K1 activation was also suppressed upon CPO treatment in both prostate and breast cancer cells. Our data clearly indicates that the inhibition of PI3K/AKT/mTOR/S6K1 signaling cascade by CPO leads to the suppression of cell proliferation in human prostate carcinoma PC-3 and breast carcinoma MCF-7 cells.

3.4. CPO induces ERK, JNK, and p38 activation in human prostate and breast cancer cells

We next performed Western blot analysis to determine whether CPO can induce the activation of MAPK cascades including JNK, ERK and p38 MAPK in tumor cells. As shown in Fig. 2B, CPO substantially induced the activation of ERK kinase at a concentration as low as 10 μM, and slightly upregulated the phosphorylation of JNK and p38 MAPK in the prostate and breast cancer cells.

3.5. CPO causes loss of mitochondrial membrane potential

The collapse of the mitochondrial membrane potential causes mitochondrial dysfunction leading to swelling, cytochrome c release, and apoptosis. Therefore, we next examined whether CPO induces changes in the mitochondrial membrane potential. As shown in Fig. 3A and B, the accumulation of Rh123 in the mitochondria was notably reduced on

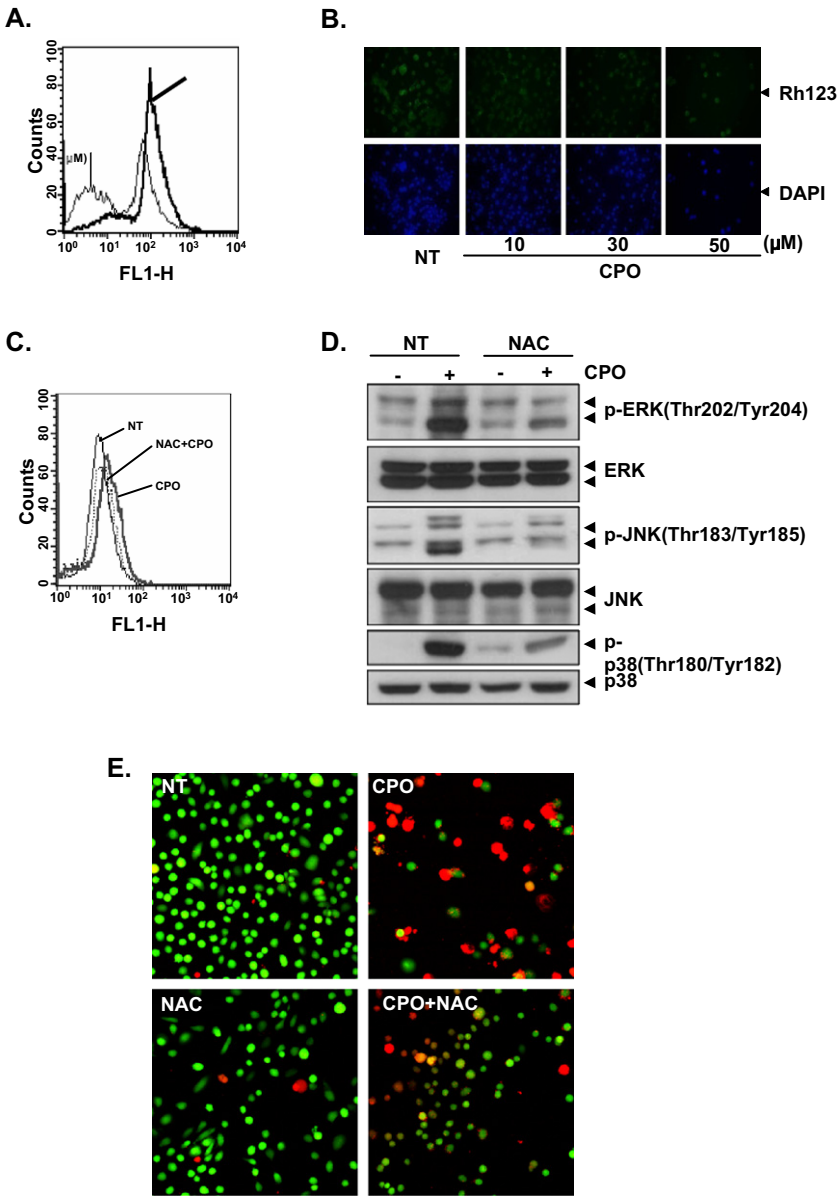

Fig. 3. CPO induced apoptosis through a loss of mitochondrial membrane potential and increased ROS production in tumor cells. After treatment with CPO for 24 h, the cells were incubated with Rhodamine123 (Rh123) and then analyzed by a flow cytometry (A) or under a fluorescence microscope (B) as described in Materials and Methods. (C) Cells were treated with CPO in the absence or presence of 5 mM NAC for 24 h. Cells were incubated with 50 μM DCFH-DA for 5 min at 37 °C. Cells were collected and DCF fluorescence was measured by flow cytometry. (D) CPO was treated in the presence or absence of N-acetyl cysteine (NAC) for 6 h. Then equal amounts of lysates were analyzed by Western blot using antibodies against p-ERK (Thr202/Tyr204), ERK, p-JNK (Thr183/Tyr185), JNK, p-p38 (Thr180/Tyr182), and p38. (E) Cells were treated with CPO in the absence or presence of NAC for 24 h. The cells were stained with a Live/Dead assay reagent for 5 min and then analyzed under a fluorescence microscope as described in experimental procedures. The results are representative of three independent experiments.

CPO treatment as compared with the non-treated cells. These results suggest that CPO induces both early and late apoptosis accompanied with mitochondrial dysfunction in prostate cancer cells.

3.6. CPO induces ROS generation in prostate cancer cells

We further investigated whether the generation of intracellular ROS is partly responsible for the apoptosis induced by CPO in prostate cancer cells. Fig. 3C shows the flow cytometric analysis of PC-3 cells treated with CPO in the presence or absence of antioxidant NAC and stained with DCFH-DA. Treatment with CPO resulted in a high increment in DCF fluorescence intensity when compared to vehicle-treated control cells. Interestingly, pretreatment of cells with broad spectrum antioxidant NAC was found to completely block the CPO-induced increase in DCF fluorescence in the tumor cells.

3.7. NAC blocks the activation of ERK, JNK, and p38 MAPK signaling proteins induced by CPO

Next, we explored the potential role of ROS in CPO-mediated ERK, JNK, and p38 MAPK activation in prostate cancer cells. Whole cellular lysates of PC-3 cells treated with CPO (50 μM) for 6 h with or without pretreatment of NAC were analyzed for p-ERK, p-JNK, and p-p38, respectively. Results showed that the pretreatment of NAC drastically prevented

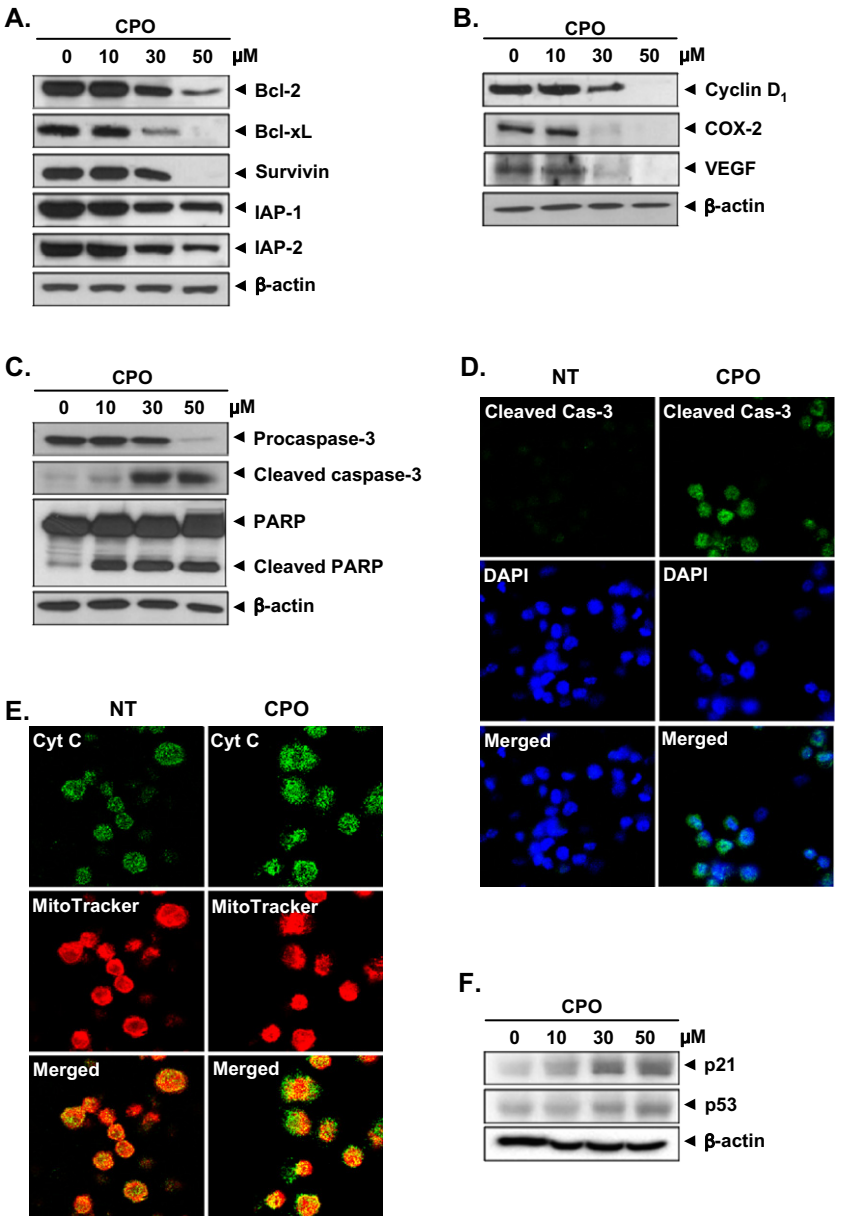

Fig. 4. CPO down-regulated the expression of various proliferative, anti-apoptotic, metastatic, and angiogenetic gene products, and induced apoptosis via caspase-3 activation and cytochrome c release. PC-3 cells were treated with indicated concentrations of CPO for 24 h. Thereafter, equal amounts of lysates were analyzed by Western blot analysis using antibodies against Bcl-2, Bcl-xL, Survivin, IAP-1, IAP-2, Cyclin D_1, COX-2, VEGF, procaspase-3, Cleaved caspase-3, and PARP (A-C). β-actin was used as a loading control (bottom panel). Cleaved caspase-3 (D) and cytochrome c release (E) was detected using immunofluorescence assay. MitoTracker (Red) was used as a mitochondria marker. (F) PC-3 cells were treated with indicated concentrations of CPO for 24 h. After then, equal amounts of lysates were analyzed by Western blot analysis using antibodies against p21and p53. β-actin was used as a loading control (bottom panel).

the activation of these protein kinases by CPO (Fig. 3D). In addition, NAC attenuated CPO-induced apoptosis as observed by Live/Dead assay (Fig. 3E). These results indicate that ROS generation may mediate the observed effects of CPO on MAPKs activation as well as apoptosis in PC-3 cells.

3.8. CPO down-regulates expression of various proteins involved in apoptosis, proliferation, metastasis, and angiogenesis

Because bcl-2, bcl-xL, survivin, IAP-1, and IAP-2 have been implicated in apoptosis and mitochondrial dysfunction, and act downstream to PI3K/AKT/ mTOR/S6K1 signaling cascade, we next examined the effects of CPO on the constitutive expression of these proteins. We found that CPO suppressed the expression of anti-apoptotic gene products in a concentration-dependent manner (Fig. 4A). Also, CPO repressed cell cycle protein (Cyclin D_1), and proteins linked with metastasis and angiogenesis (COX-2 and VEGF) (Fig. 4B). Procaspase-3 is mainly activated in the apoptotic cell via both the mitochondrial-dependent/independent pathways, and causes cleavage of PARP involved in the repair of DNA damage. Therefore, we also investigated the effect of CPO on procaspase-3 level and PARP cleavage in PC-3 cells. CPO clearly induced the cleavage of procaspase-3 as observed by the disappearance of the procaspase-3 band and appearance of its cleaved forms. Sequentially, CPO induced PARP cleavage in a concentration-dependent manner (Fig. 4C). Additionally, increased

caspase-3 activity and cytochrome c release was clearly observed after CPO treatment by FITC-conjugated immunofluorescent staining method (Fig. 4D and E). These results clearly demonstrate that CPO can induce apoptosis via the down-regulation of anti-apoptotic, proliferative, metastatic, angiogenetic proteins via caspase-3 activation in tumor cells.

3.9. CPO induces the expression of both p53 and p21 in PC-3 cells

p53 is important in multi-cellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor that is involved in preventing development of cancer [30]. The expression of p21 is controlled by the tumor suppressor protein p53 [31]. The cyclin-dependent kinase inhibitor p21 is prototypical member of the Cip/Kip family of cyclin-dependent kinase inhibitors. It negatively modulates cell cycle progression by inhibiting the activities of cyclin E/CDK2 and cyclin D/CDK4 complexes and blocks DNA replication by binding to proliferating cell nuclear antigen [32]. We found that CPO induced the expression of both p53 and p21 in a concentration-dependent manner in prostate tumor cells (Fig. 4F).

3.10. Apoptotic effects of CPO is enhanced in combination with pharmacological PI3K/AKT blockers

Furthermore, we investigated whether the effects of CPO on cytotoxicity and PARP cleavage were enhanced by the combination with pharmacological PI3K/AKT inhibitors (wortmannin and AKT inhibitor IV). As shown in Fig. 5A, the combination potentiated cytotoxicity when compared with CPO alone in PC-3 cells, and the PARP cleavage was also synergistically increased by the combination of CPO with these inhibitors (Fig. 5A, *bottom*). Whether AKT inhibition by the combination treatment is consistent with PARP cleavage was examined by analyzing the effect of CPO on the constitutive AKT, mTOR, and S6K1 activation in PC-3 cells. As shown in Fig. 5B, treatment with CPO, wortmannin, and AKT inhibitor IV alone completely inhibited constitutive AKT activation, but had minimal effect on mTOR and S6K1 activation. Interestingly, mTOR and S6K1 activation were completely blocked by the combination of CPO with diverse pharmacological inhibitors. These data imply that CPO is not only a potential AKT inhibitor, but can also act through modulation of multiple signal transduction pathways to induce apoptosis in tumor cells.

3.11. Dual regulation of AKT and mTOR proteins by CPO

To further understand how CPO modulates various cell signaling pathways, the potential effects of CPO on PI3K/AKT/mTOR/S6K1 signaling cascade were compared to those of an AKT inhibitor (AKi), a mTOR inhibitor (rapamycin), and dual PI3K/mTOR inhibitor (NVP-BEZ235). Western blot analysis showed that AKi and rapamycin were the most potent in inhibiting AKT and mTOR activation, respectively (Fig. 5C). However, NVP-BEZ235 and CPO could broadly block all the four kinases in PI3K/AKT/mTOR/S6K1 signaling axis when compared with AKi and rapamycin, thereby indicating that CPO can regulate multiple molecular targets and signaling pathways in tumor cells.

4. Discussion

Even though CPO has been known to suppress the proliferation of a wide variety of cell types and exert anti-inflammatory effects, the exact mechanism of action of CPO has not yet been elucidated. The aim of our study was to investigate the effects of CPO on the PI3K/AKT/mTOR/S6K1 and MAPKs signaling, gene products, and cellular responses. Here, we observed that CPO not only exerted significant anti-proliferative and apoptotic effects, but it also suppressed constitutive PI3K/AKT/mTOR/S6K1 activation and induced ROS-mediated MAPKs activation in human prostate and breast cancer cells. Consequently, this sesquiterpene down-regulated various gene products involved in cell proliferation, anti-apoptosis, and metastasis. We also clearly demonstrate that CPO can target both PI3K/AKT/mTOR/S6K1 and MAPK signaling pathways and induce apoptosis through caspase-3 activation and cytochrome c release in diverse tumor cells.

Although previous studies have shown that guava leaves, which are one of the major sources of CPO can suppress the growth in two other human prostate cancer cells namely DU145 and LNCaP cells, our study is the first report to specifically examine AKT/mTOR/S6K1, MAPKs signaling, gene products, and the cellular responses in both human prostate cancer PC-3 and breast cancer MCF-7 cells. Recently, Chen et al. have demonstrated that guava leaves can induce apoptosis through the inactivation of AKT in LNCaP cells [33]. However, our findings more specifically indicate that constitutive PI3K/AKT activation was abrogated by CPO. One possible explanation for this could be a significant difference in sensitivity to cell growth signals in PC-3 and LNCaP cells. Unlike LNCaP cells, the androgen-independent prostate cancer PC-3 cell line does not express detectable levels of AR mRNA [34] or hPAP mRNA in Northern blot analysis [35] and are unable to secrete hPSA or hK2 [36]. Also, it has been previously reported that the level of AKT activation is drastically enhanced in androgen-independent cells as compared with the androgen-dependent cells [37]. Importantly, this is the first report to show the effects of CPO on PI3K/AKT/mTOR/S6K pathways in prostate and breast cancer cells. We found that CPO suppressed the expression of phospho-AKT (Ser473) in a concentration-dependent manner in PC-3 and MCF-7 cells, which further indicates the possibility that CPO could interfere with TORC2 (the mTOR-rictor complex that phosphrylates AKT at Ser473). AKT is also reported to regulate the master transcription factor NF-κB through the phosphorylation of p65 to enhance the transcriptional activity of NF-κB [38]. Furthermore, AKT is also positively involved in COX-2 expression in the TLR4 signaling pathway [39]. Choi et al. showed that guava leaves can indeed inhibit LPS-stimulated iNOS and COX-2 production via the down-regulation of NF-κB activation [40]. NF-κB activation is known to regulate the expression of various cell survival, proliferative, metastatic, and angiogenic gene products [41]. Although, the potential effect of CPO on the modulation of NF-κB signaling cascade is not the focus of the present study and requires additional investigation, it is clear from our results that the inhibition of the PI3K/AKT/mTOR/S6K pathways may contribute to the anti-cancer effects of CPO.

Our results also indicate, for the first time, that CPO suppressed constitutive mTOR (Ser2448) activation in PC-3 and MCF-7 cells. There are many reports in literature indicating that the aberrant activation of mTOR is closely associated with tumorigenesis [42–44]. Recently, specific mTOR inhibitors have shown great promise in clinical trials for the treatment of various malignant tumors [45]. This, it is possible that targeting mTOR signaling pathway with a range of agents derived from natural sources can reveal a number of potential candidates both for the prevention and treatment of cancer. We also found that CPO inhibited constitutive S6K1 (Thr421/Ser424) activation, which is a direct downstream target of mTORC1 (mTOR, Gβ1, and raptor).

We also investigated how CPO induces ERK, JNK, and p38 MAPK activation in tumor cells. Several reports indicate that

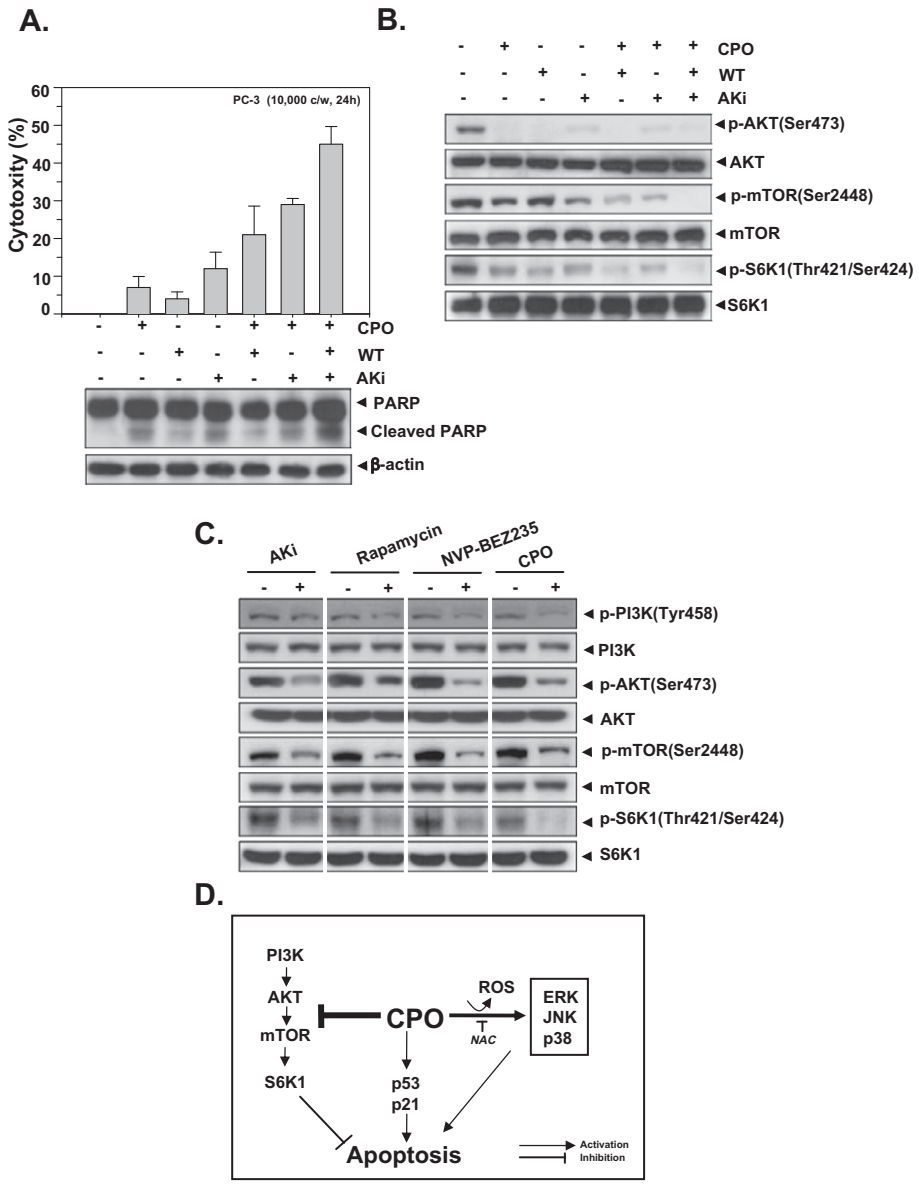

Fig. 5. CPO potentiated the apoptotic effects of pharmacological PI3K/AKT blockers in tumor cells. (A) After PC-3 cells were seeded onto 96-well plates or 6-well plates, the cells were treated with CPO (30 μM), wortmannin (WT, 0.4 μM) or AKT inhibitor IV (AKi, 1 μM) and the indicated combination of CPO with pharmacological PI3K/AKT blockers for 24 h and then subjected to the MTT assay or Western blot analysis using antibodies against PARP and β-actin. (B) PC-3 cells were treated with indicated combination of CPO (30 μM), wortmannin (0.4 μM) or AKT inhibitor IV (1 μM) for 6 h. Then the equal amounts of lysates were analyzed by Western blot analysis using antibodies against p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-S6K1 (Thr 421/Ser424), and S6K1. (C) PC-3 cells were treated with the AKT inhibitor IV (AKi, 1 μM), Rapamycin (20 nM), NVP-BEZ235 (50 nM), or CPO (30 μM) for 6 h and then analyzed by Western blot analysis using antibodies against p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-S6K1 (Thr 421/Ser424), and S6K1. (D) Schematic diagram showing the effects of CPO on PI3K/AKT/mTOR/S6K1 and MAPKs signaling pathways and apoptosis.

most anti-cancer agents or ionizing radiation that can induce ROS generation can also concurrently activate MAPK pathways in multiple cell types [22–24,46]. In addition, thymoquinone and isointermedeol, derived from various essential oils, have been reported induce apoptosis through the generation of ROS in different tumor cells [27,47]. Our present findings also provide evidence of mitochondrial involvement in the generation of ROS by CPO. The generation of ROS in response to CPO was further supported by the finding that pretreatment with NAC blocked the ROS-mediated MAPK activation and prevented CPO-induced apoptosis in cancer cells. Our results are in agreement with a previous report that guava leaves can induce apoptosis through the inactivation of phospho-AKT, activation of phospho-p38, phospho-ERK in human prostate LNCaP cells [33]. Thus, it is very likely that the induction of MAPK activation can contribute to the suppression of tumorigenesis in part upon CPO treatment in tumor cells. Interestingly, a

recent study indicated that CPO contains reactive exocyclic methylene and epoxide functional groups, which can enable it to form covalent bonds with sulfhydryl and amino groups of various proteins and DNA bases [48]. Thus, the presence of these key functional groups can account for CPO's enormous potential to modulate multiple signal transduction cascades in tumor cells as also reported in the present study.

We further found that the expression of various gene products involved in tumor initiation and promotion was also suppressed by CPO and it may explain the previously-reported anti-cancer and anti-inflammatory effects of CPO. These include proliferative (cyclin D1), anti-apoptotic (bcl-2, bcl-xL, survivin, IAP-1, and IAP-2), metastatic (COX-2), and angiogenic (VEGF) gene products. AKT signaling through mTOR is an important mechanism of oncogenesis that can protect cancer cells from apoptosis and drug resistance *in vivo* [49]. Thus, the suppression of PI3K/AKT/mTOR/S6K activation by CPO could facilitate downregulation of various cell survival genes and lead to apoptosis in tumor cells. Activated AKT can also exert anti-apoptotic effects, positively regulate NF-κB transcription, modulate angiogenesis, promote tumor invasion/metastasis, and antagonize cell cycle arrest [50]. Consistent with this evidence, we found that CPO was able to clearly suppress COX-2 and VEGF expression at a low concentration of 30 μM. Thus, it is possible that the suppression of COX-2 and VEGF is the potential link for the inhibition of metastasis, angiogenesis, and invasion by CPO. Since bcl-2 and bcl-xL differentially protect human prostate cancer cells from the induction of apoptosis [51], their down-regulation could contribute to the ability of CPO to induce apoptosis in tumor cells. Zhang et al. have indeed shown that survivin mediates resistance to anti-androgen therapy in prostate cancer [52].

We also observed that CPO inhibited both the proliferation and the expression of cyclin D1, which is consistent with previous reports that both cyclin D1 [53], as well as p70S6K are required for cell growth and G1 cell cycle progression in tumor cells [54]. We found that the expression of p53 and $p21^{WAF1/CIP1}$ tumor suppressor proteins, which can block the cell cycle at the G1 phase by inhibiting the activities of cyclin/cyclin dependent kinases (CDK) complexes, was also upregulated upon CPO treatment. We further observed that CPO could block constitutively survivin expression, suggesting that the use of CPO can lead to the development of a novel strategy to enhance sensitivity to androgen ablation therapy. In addition, we found that this sesquiterpene can also significantly potentiate the apoptotic effects of specific AKT inhibitors, and it is very likely that this potentiation is mediated through the suppression of anti-apoptotic gene products regulated by PI3K/AKT/mTOR/S6K1 signaling.

Overall, our results indicate for the first time that CPO can inhibit constitutive PI3K/AKT/mTOR/S6K1 signaling cascades, induce ROS-mediated MAPKs activation, and lead to the induction of apoptosis through the down-modulation of gene products that mediate tumor cell survival, proliferation, metastasis, and angiogenesis in human prostate and breast cancer cells. However, further studies are needed in animals to validate these findings for the therapeutic use of this agent in humans.

5. Conflict of interest

None declared.

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