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The Combination of Artesunate and Paclitaxel in 1:1 Ratio Induces Apoptosis and Morphology Change on Human Prostate Cancer Cell Lines

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Abstract

The combination of Artesunate (ART) and Paclitaxel (PTX) in two human prostate cancer (PCa) cell lines (PC-3 and LNCaP) was evaluated to investigate the effects on proliferation, apoptosis and morphological changes. The half maximal inhibitory concentration (IC_{50}) values that were observed by ART and PTX on both LNCaP and PC-3 cell lines at 72-and 120-hour exposure were used to assess these effects. Early and late apoptosis was detected in Annexin V-FITC/PI assay revealed a shift in population of cells towards early and mid-apoptosis with ART + PTX than with ART and PTX individually. More effects were observed on LNCaP cell lines at both 72-hour and 120-hour exposure. The results for the Caspase 3/7 activity assay showed shift of viable population in all induced samples compared to control. Morphological changes occurred in both cell lines; this was validated in qualitative assessment when examined under the inverted microscope. These findings indicated that ART + PTX suppressed PCa cell proliferation in a dose- and time-dependent manner.

Keywords

Combination, Prostate Cancer, Artesunate, Paclitaxel, Anticancer Activities

1. Introduction

Prostate cancer is categorized as adenocarcinoma, defined by the abnormal development of the gland cells. In normal prostate functions, the prostate is the size of a walnut. The gland is located around the urethra at the base of the penis. The size of the prostate may change with age, in general cases it becomes much

larger as men become elderly. It functions to produce about one-third of the fluid present in semen. This fluid aids in nourishing and maintaining a high pH for sperm survival [1]. The cause of prostate cancer continues to be unclear although inflammation is found to be the generalization that leads to cancer. Inflammation is a key driver of prostate cancer metastasis and therapeutic resistance. Chronic inflammation has been identified as a major cause of approximately 20% of human cancer cases. Environmental exposure to bacterial, viral infections, dietary habits, hormonal influences, physical injury, exposure to mutagenic agents, and genetic variation predispose the prostate gland to inflammation. An inflammatory response is coordinated with an elevated expression of inflammatory cytokines. Chronic inflammation in the prostate can alter the tumor microenvironment suitable for cancer progression, proliferation, cell survival, metastasis spread and resistance to therapeutic agents. Inflammation in prostate progression can be detected in histology exams in which lesions containing activated inflammatory immune cells infiltrate with the peripheral zone of the prostate [2]. Every year, 20.7 out of 100,000 men die of prostate cancer and 11.11% of men will be diagnosed with prostate cancer at some point [3]. Due to the growth and aging of the global population, it was estimated the global epidemiology of prostate cancer is increasing by approximately 1.7 million new cases per year and about 499,000 mortalities are expected by 2030 [4]. In the US, there are about 288,300 new cases and approximately 34,700 deaths from prostate cancer have been estimated by the American Cancer Society for 2023 [5]. The Food and Drug Administration (FDA) is constantly seeking prospective anticancer agents to be approved for various cancers [6].

Artesunate (ART) (shown in **Figure 1**) is isolated and extracted from dried leaves and buds of the Wormwood plant (*Artemisia annua L*) produced in southwest China. It is considered a semi-synthetic derivative, water soluble, sesquiterpene lactone compound that contains an endoperoxide radical without any nitrogen atoms in its structure. ART is primarily an antimalarial drug used since 1973 for malaria caused by *Plasmodium falciparum* and cerebral malaria, although it has been shown to have some cytotoxic properties. In the 1990's, Artemisinin was first reported to have anticancer effects by *in vitro* and *in vivo* studies in a variety of tumor cells such as leukemia, colorectal cancer, melanoma, breast and prostate cancer, among others. This can make a promising repurposed agent for the treatment of prostate cancer. New insight brought by growing evidence showed that Artesunate is expected to be a new class of antitumor drug of wide spectrum. The main active anticancer sites of ART are the same as the antimalarial sites [7] [8]. All derivates focus on keeping the peroxide bridge and modification of other portions of compound. The ART derivatives target cell membranes as the principal target sites. They have not only been shown to induce apoptosis but play a role in cell swelling. The cytomembrane is destroyed in the process and the membrane permeability is altered which results in death of cells [9].

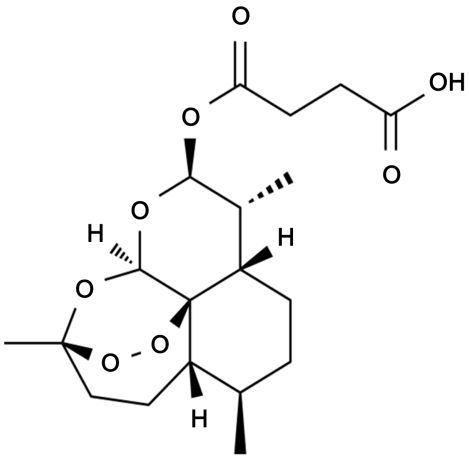

Figure 1. Chemical structure of Artesunate (ART).

Ovarian cancer cell lines demonstrated a strong induction of reactive oxygen species (ROS) and reduced proliferation when treated with ART. This caused damage to membranes, autophagy and apoptosis, including inhibiting angiogenesis of cancer cells. Additionally, cell cycle arrest is caused by the change of expression and activity in several regulatory enzymes [10]. In Ishikawa endometrial cancer cells, ART has induced G1 cell cycle arrest and downregulated cyclin-dependent kinase 4 gene expression which is seen to play a role in cancer progression [11].

Paclitaxel (PTX) (shown in **Figure 2**) on the other hand, is a taxane-class drug used for the treatment of prostate cancer. It is derived from the bark of the Pacific Yew Tree (*Taxus brevifolia*). Although registered and approved as a cancer therapy drug by FDA for the treatment of prostate cancer, it is limited due to the amount of serious side effects and recent occurring drug resistance. PXT's mechanism of action is to induce cell cycle arrest and apoptosis in most cancer cells. It works by binding and stabilizing the β-tubulin microtubule polymer interfering with the microtubule assembly of mitosis and prolonged activation in the G2/M phase of the cell cycle [12].

Some cancers, such as lung and breast cancers may demonstrate resistance to paclitaxel through various mechanisms such as mutations of caspases in prostate carcinoma PC-3 cells. Due to paclitaxel cancer resistance, literatures support the use of adjuvant treatment and/or combination therapy to inhibit autophagy caused by paclitaxel-resistance. Reports showed that the use of chloroquine and paclitaxel reverts paclitaxel resistance in non-small lung adenocarcinoma via ROS [13] [14]. The enhancement of paclitaxel-induced apoptosis through the use of adjuvant treatments and/or combination therapy has been shown for various cancers. Artesunate in combination with Paclitaxel could provide insight for apoptosis enhancement through similar mechanisms and reverse paclitaxel resistance through expression of proapoptotic proteins. ROS activity can potentiate the effect of paclitaxel thus increasing apoptosis rate in cell cycle arrest G0/G1.

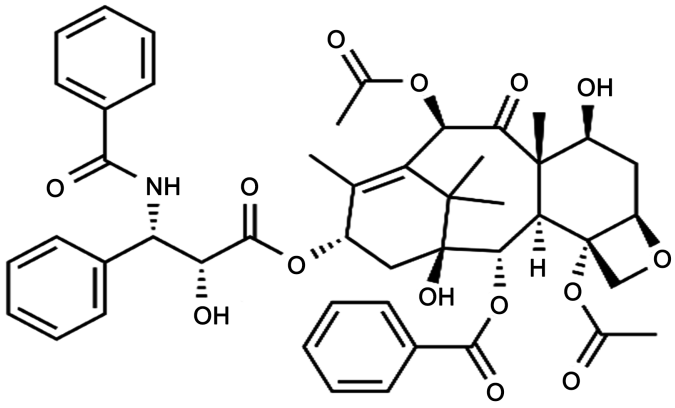

Figure 2. Chemical structure of Paclitaxel (PTX).

The aim of this study was to investigate the induction of apoptosis by combining ART and PTX at a 1:1 ratio of the determined individual IC_{50} values of ART and PTX on both the androgen-dependent (LNCaP) and androgen-independent (PC-3) human prostate cancer cell lines. Assays were performed using Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) and Caspase 3/7 activity in a time-dependent manner at 72- and 120-hour using the flow cytometer.

2. Materials and Methods

2.1. Cytotoxicity Assessment of ART and PTX

The MTT cell proliferation assay was used to assess the cytotoxic properties of ART and PTX on LNCaP and PC-3 Cell lines after 72- and 120-hour exposure. LNCaP and PC-3 cell lines, purchased from American Type Culture Collection (ATCC) were cultured and maintained in RPMI 1640 media supplemented with 10% Fetal bovine serum (FBS, penicillin-streptomycin, sodium pyruvate, glutamine and non-essential amino acids at 37˚C in 5% CO_2 in a humidified incubator as previously reported [15]. Media were changed every 2 - 3 days until the cell lines reached about 70% - 80% confluency. Cells were trypsinized with 0.5% trypsin-EDTA. Cell lines were seeded into 96-well plates. ART and PTX were administered to both cell lines at different concentrations of 10^{-3} - 10^{2} µM. Cell viability was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, a reducing agent. The MTT assay was performed after 72- and 120- hour exposure of the individual cell line to each concentration. The MTT assay protocol has been reported in [16].

2.2. Annexin V-Fluorescein Isothiocyanate/Propidium Iodine (FITC/PI) Apoptosis Assay

This assay was carried out using TACS® Annexin V-FITC Apoptosis Detection Kit (Cat#4839-01-K) following manufacturer's protocol. Briefly, PC-3 and LNCaP cell lines were treated with ART, PTX and ART: PTX (1:1) seeded at a concentration of 1×10^{5} cells/well overnight in a Corning Cellbind 6-well plate. Cells

were washed by resuspending in 500 µl of cold 1× PBS and centrifuged at 300 ×g for 6 minutes at room temperature to collect the pellet. The pelleted cells were gently resuspended in the Annexin V reagent (composed of 10× binding buffer, TACs annexin V-FITC, distilled water and PI). Annexin V incubation (conjugate with fluorescein isothiocyanate (FITC) were added and incubated at 37˚C for 15 min followed by the addition of a binding buffer. Cells were washed with phosphate-buffered saline (PBS). The cells were analyzed for apoptosis and morphology based on cell membrane changes and fluorescence intensity using the flow cytometer at 494 nm and emission at 519 nm (Guava easyCyte single loader, USA) [17].

2.3. Caspase 3/7 Assay

LNCaP and PC-3 cell lines were seeded at 1×10^5 cell/well in a Corning Cellbind 6-well plate and treated with ART, PTX and ART: PXT ratio concentration for 72 h. Then, caspase activity was determined according to the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Catalog# C10427, Invitrogen, USA) manufacturer's protocol. Equal volumes of 1 mL samples contained 1 µL of the CellEvent Caspase-3/7 green detection reagent was added and incubated at room temperature for 30 min at 37˚C followed by the addition of 1 µL of 1 mM SYTOX™ AADvanced™ dead stain cell solution for 5 minutes at 37˚C. The intensity was analyzed for both cell lines at 488 nm excitation using the flow cytometer (Guava easyCyte single loader, USA).

3. Results and Discussion

MTT assay was used to determine the anti-carcinogenic activities of ART, PTX and ART + PTX on both androgen-sensitive LNCaP and androgen-insensitive PC-3 prostate cancer cell lines at 72-and 120-hour exposure. The result revealed dose-dependent and time-dependent cytotoxicity on both cell lines. In Annexin V-FITC/PI assay, the combination of ART and PTX (ART + PTX) at ratio 1:1 showed to have an increase of early and late apoptotic effect than in the single drug groups when examined on both LNCaP and PC-3 cell lines. As shown in **Figure 3** and **Figure 4**, there was a shift in population of cells towards the upper right quadrants that indicate early apoptosis moving towards a late stage of apoptosis in a time dependent manner from 3 to 5 days. In a single ANOVA factorial analysis (not shown), the 72- and 120- hour of treated PC-3 and LNCaP cell lines gave a *P value of* <0.05. In a Tukey analysis (not shown), 72-hour annexin early apoptosis of PC-3 and LNCaP treated indicated a significant difference within treated groups and between untreated groups while the 120-hour annexin early apoptosis of LNCaP did not reveal a significant difference between ART and PTX, but there was a significant difference between ART + PTX when combined. In a Tukey analysis, 120-hour annexin early apoptosis of PC-3, there was not a significant difference between ART + PTX and ART, although there was a higher average in ART + PTX and ART. This suggests that ART combination with PTX significantly induces a significant degree of apoptosis in PCa cell lines.

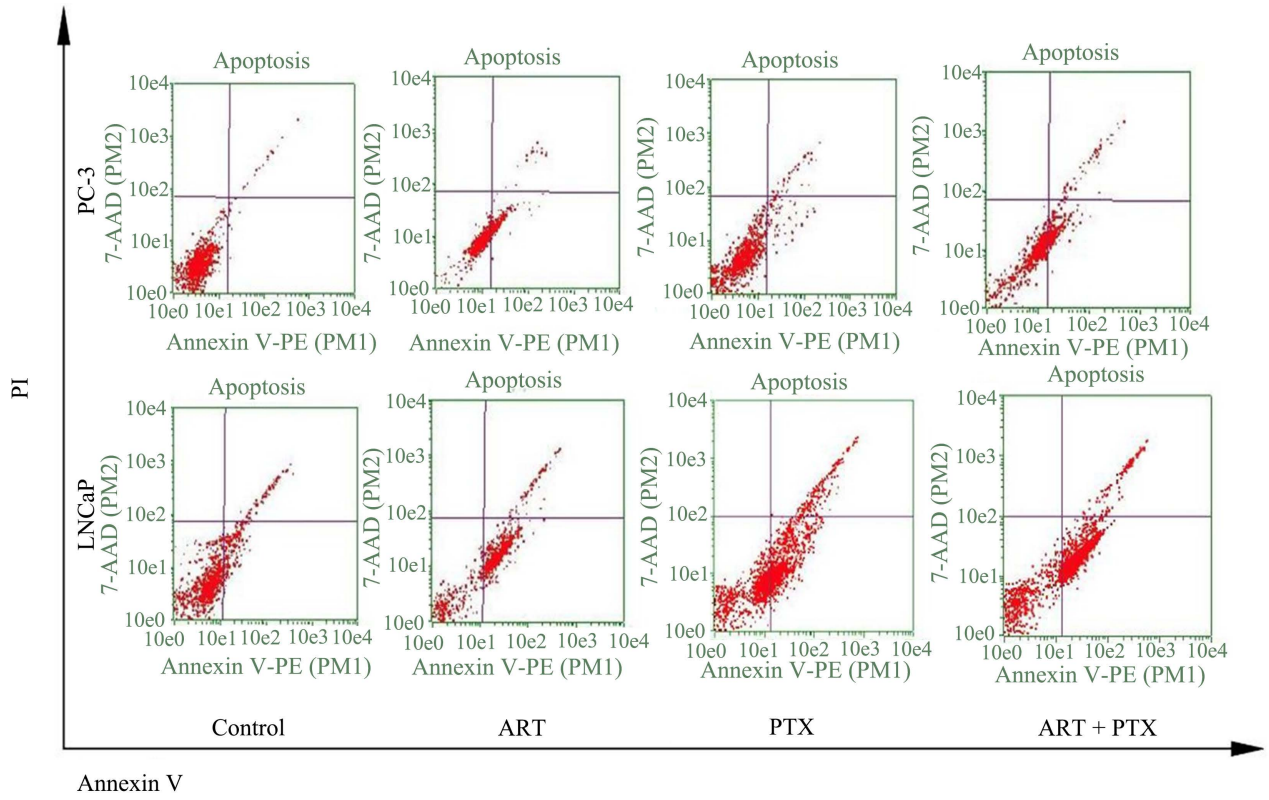

Figure 3. Annexin V-FITC/PI on LNCaP & PC-3 at 3-day exposure.

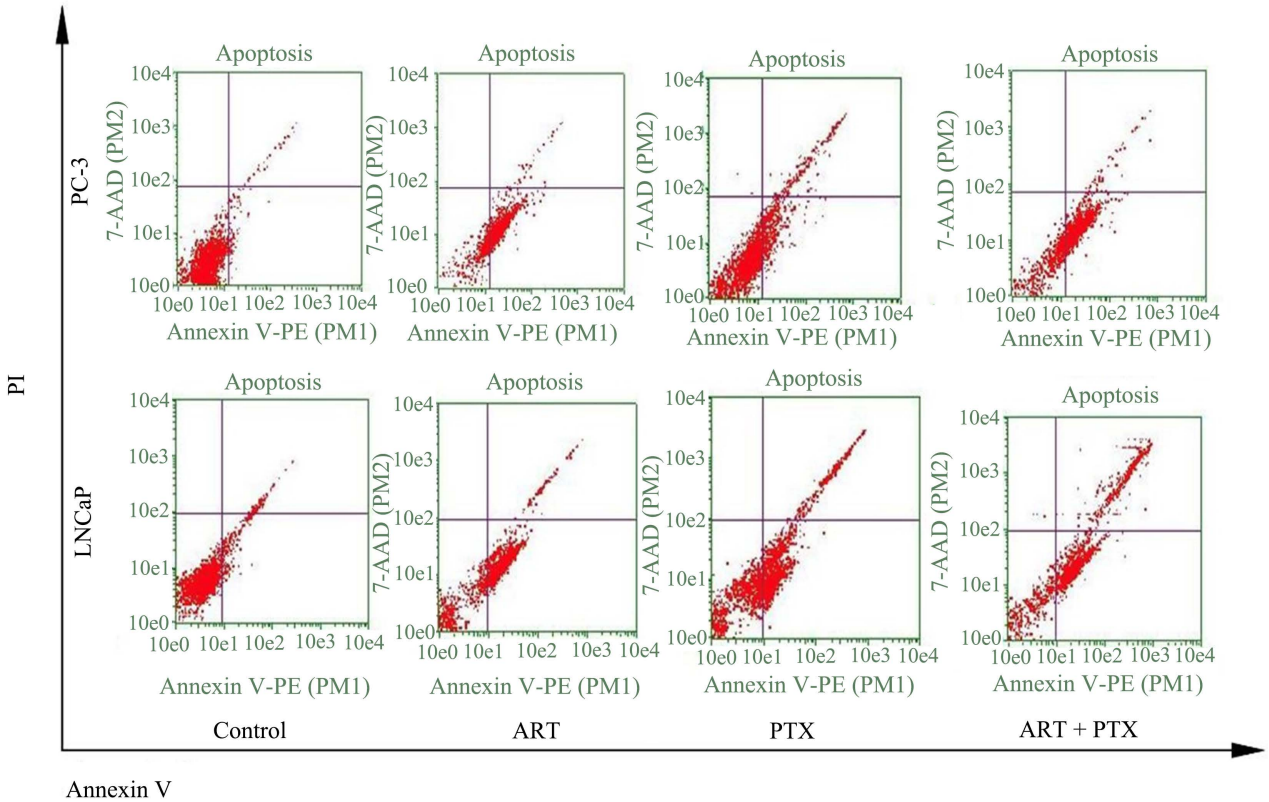

Figure 4. Annexin V-FITC/PI on LNCaP & PC-3 at 5-day exposure.

The release of *cytochrome c* from the mitochondria activates down-stream caspase molecules that lead to apoptotic cell death. A flow cytometric caspase assay measured by examining the green, fluorescent intensities of caspase-3/7 activities of ART, PXT and ART + PTX on both LNCaP and PC-3 cell lines at 72- and 120-hour exposure were assessed. Results (Figure 5 and Figure 6) indicated there was higher caspase 3/7 activity in ART + PTX compared to control and other inducers in both cell lines at 72 and 120 hours. Triplicate trials in inducers and control had a *P value* < 0.05 in all experiments. There is a right shift in population that probably indicates induction of caspase 3/7 protein. According to a Tukey test, control and inducers were significantly different from one another in PC-3 at 72-hours. In LNCaP, ART and PTX were not significantly different but ART + PTX were significantly different from ART, PTX, and control at 72 hours. In PC-3 at 120 hours, there is not a significant difference between ART + PTX and ART. In LNCaP, at 120 hours, ART and PTX were not significantly different but ART + PTX were significantly different from ART, PTX, and control.

Subsequently, ART, PTX and ART + PTX-induced growth inhibition in PCa cells was visualized by using microscopy. Cells cultured without these reagents exhibited characteristic normal growth and shape compared to cells treated with ART, PTX and ART + PXT (Figure 7 and Figure 8). The controls for both cell lines exhibited confluency. Annexin V fluorescence intensities accompanied by

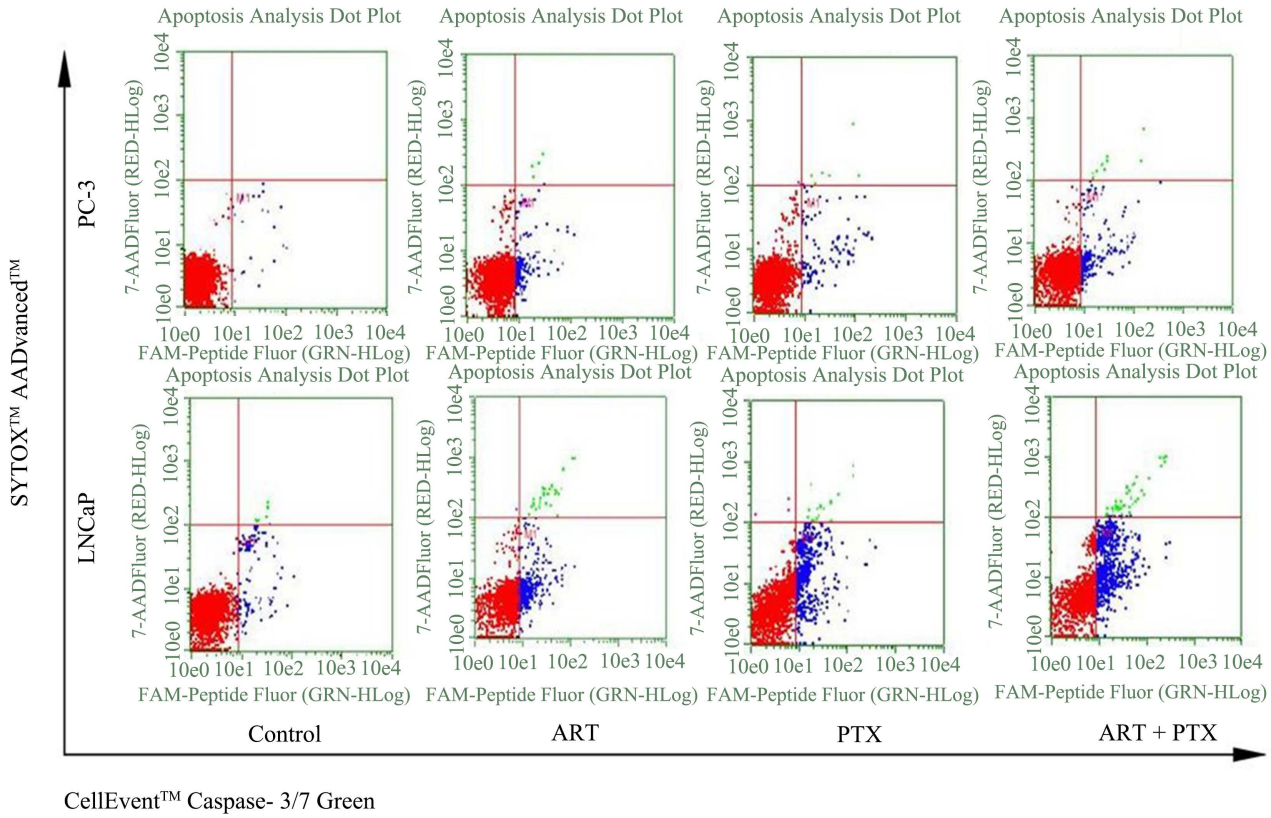

Figure 5. Caspase 3/7 Detection after 72-hour exposure.

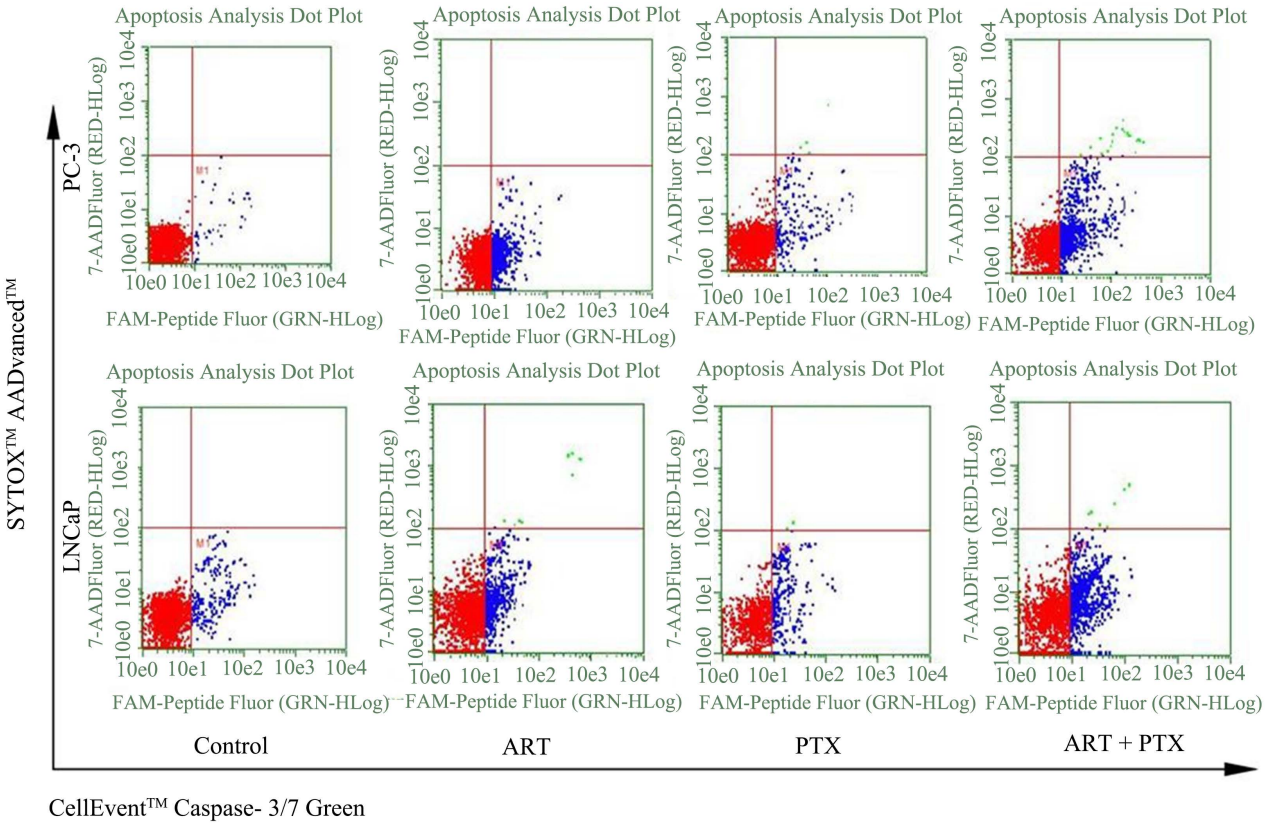

Figure 6. Caspase 3/7 Detection after 120-hour exposure.

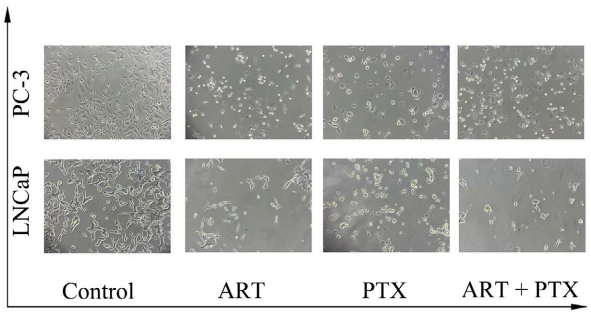

Figure 7. Cell Morphology (inverted microscope) examination after 3-day exposure of ART, PTX and ART + PTX (1:1 ratio).

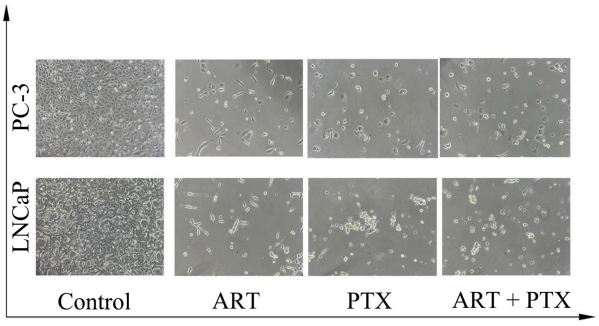

Figure 8. Cell Morphology (inverted microscope) examination after 5-day exposure of ART, PTX and ART + PTX (1:1 ratio) on both LNCaP and PC-3 Cell Lines.

cell shrinkage was observed in ART + PTX treated compared to ART and PXT solely for PC-3 and LNCaP cells. Cell shrinkage was validated in a qualitative assessment when examined under the inverted microscope. This finding suggests that ART in combination with PTX suppressed cell proliferation in a time-dependent manner.

In summary, early to late apoptosis was detected using Annexin-FITC/PI and Caspase 3/7 activity on LNCaP and PC-3 cell lines. It was observed that ART + PTX had the highest degree of apoptotic cells as seen in both assays. This was validated by microscopy examination using the inverted microscope. Microscopic images revealed morphological changes with pseudopodia shrinkage following the treatment of ART, PTX, and ART + PTX indicating apoptosis compared to control in both prostate cancer cell lines. Cell shrinkage is a common indicator of apoptosis. In a time-dependent manner, there was more cell shrinkage and less cell adherence in ART + PTX at 120-hour compared to ART + PTX at 72-hour-exposure in both cell lines. These findings support the idea that ART + PTX induces a higher degree of apoptosis and caspase activity than ART and PTX solely on LNCaP and PC-3 cell lines. This study could provide promising insights into a novel and potential therapeutic strategy for the treatment of cancers (prostate) and open new opportunities for prevention of PTX resistance and probably prevention of metastasis in various cancers.

Future work includes the combination studies of ART + PTX on normal cell lines to validate the cytotoxic effects on target prostate cancer, detection of the expression levels of proapoptotic and antiapoptotic proteins such as Bax, Bcl-2 and Bcl-xL using Western blot [18]. In addition, further studies on cell cycle arrest at G2/M to validate that ART can potentiate PTX resistance and the mechanism of action.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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