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# LYSOPHOSPHATIDIC ACID MODIFIES THE RESPONSE OF PC3 PROSTATE CANCER CELLS TO CHEMOTHERAPEUTICS

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**Abstract – *Objective:*** *Prostate cancer is the most frequently diagnosed cancer among men. Docetaxel, estramustine, and mitoxantrone are commonly used chemotherapy agents for the treatment of prostate cancer. However, lysophosphatidic acid (LPA), a biologically active glycerophospholipid derivative, induces proliferation and inhibits apoptosis in prostate cancer cells. The aim of this study was to investigate the effects of LPA against cell toxicity of docetaxel, estramustine and mitoxantrone.*

***Materials and Methods:*** *Prostate carcinoma PC3 cells were separately treated with docetaxel, estramustine and mitoxantrone in combinations with LPA. BrdU incorporation assay was used to assess the cell proliferation. Besides, colony forming ability of cells were measured by staining with crystal violet. The ratio of apoptotic cells was also detected by flow cytometry.*

***Results:*** *All the chemotherapeutic drugs decreased the proliferation and colony formation of PC3 cells whereas these parameters were found to be significantly increased in the cells treated with LPA alone. Treatment of drugs together with LPA increased cell proliferation and colony formation compared to the treatment of with drugs alone. Also, LPA was seen to modify the apoptotic effects of docetaxel, estramustine and mitoxantrone.*

***Conclusions:*** *Our results showed that LPA contributed to cell survival and proliferation in PC3 prostate cancer cells. LPA created a resistance against cell death induced by docetaxel, estramustine and mitoxantrone. Our study supports the idea that LPA or its signaling pathways may be a promising target for the treatment of prostate cancer and prevention of resistance to chemotherapy.*

**KEYWORDS:** *Apoptosis, Cell proliferation, Docetaxel, Estramustine, Lysophosphatidic acid, Mitoxantrone, Prostate cancer.*

## INTRODUCTION

Prostate cancer is the most common malignant neoplasm among men[1]. Radical prostatectomy and/or radiation therapy in combination with androgen suppression are recommended as standard treatment in early stage prostate cancer[2]. Chemotherapy is a suitable treatment option in patients with metastatic prostate cancer and insensitive to hormone therapy[3]. Docetaxel, estramustine and mitoxantrone are chemotherapeutic agents commonly used in the combination treatments of prostate cancer. Docetaxel, a class of taxanes, is a cytotoxic chemotherapy drug targeting beta-tubulin and shows antitumor activity by inducing apoptosis and cytotoxicity[4]. Docetaxel is shown to exhibit its apoptotic effects by decreasing antiapoptotic Bcl-2 and by increasing pro-apoptotic molecules, e.g., caspase-3, caspase-9 and Bad[5]. Es-



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tramustine, an alkylating agent, consists of estradiol and nornitrogen mustard linked with carbamate. It is used in the treatment of advanced prostate cancer due to its antineoplastic and antiandrogenic effects. As an anti-neoplastic agent, it can disrupt microtubule integrity by binding to tubulin units or microtubule-associated proteins[6,7]. Its metabolites are also known to serve as an androgen antagonist due to its binding property to androgen receptors and thereby reducing the effects of androgen hormones[8]. Mitoxantrone, an anthracendione derivative and a DNA topoisomerase II inhibitor, is one of the FDA-approved anticancer agents commonly used in the treatment of advanced prostate cancer[9]. The main problem in the treatment with chemotherapeutic drugs is drug resistance, developing especially with combined treatments.

There are several mechanisms for drug resistance, which include mutation of some cancer cells and thereby loss of sensitization to chemotherapy, efflux of drugs due to the upregulation of P-glycoprotein which serves as an export pump of substances, and inactivation of drugs by a tolerance mechanisms developed in cancer cells[10].

Lysophosphatidic acid (LPA) is known as a biologically active glycerophospholipid derivative due to its roles in a wide range of cellular processes (e.g., survival, proliferation, migration and prevention of apoptosis and senescence) through binding to G protein-coupled receptors (GPCRs; also called as LPA receptors 1–6) and activating the respective downstream signaling pathways[11,12]. LPA is produced by two enzymatic pathways. The first way is from lysophosphatidylcholine via autotaxin (ATX; has lysophospholipase D activity), a plasma enzyme. The second one is from phosphatidic acid (PA) via deacylating enzymes, phospholipase A1 or phospholipase A2[13]. LPA and its enzyme ATX are involved in different pathological conditions including cancer, lung fibrosis, liver disease and obesity. Both pathways have been suggested to be potential drug targets due to increased levels of plasma in some diseases[13]. Many studies have reported that LPA is present at high levels in the ascitic fluids and plasma of patients with prostate, ovarian, cervical, colon, endometrial, and liver cancers[14,15]. It has a role in the development of tumor angiogenesis and metastasis due to its stimulatory effects on cell growth and proliferation[16,17]. There are few detailed studies investigating the effects of LPA on chemotherapeutic agents which are used in prostate cancer. The aim of this study was to examine whether the use of LPA in combination with docetaxel, estramustine, or mitoxantrone could alter the therapeutic efficacy of these drugs in human PC3 cells. Our second objective was to compare the effects of three chemotherapeutic drugs on apoptosis and cell proliferation in the presence and absence of LPA.

## MATERIALS AND METHODS

### *Materials*

Oleoyl-L-a-lysophosphatidic acid sodium salt (LPA, Cat: L7260), Docetaxel (Cat: 01885), Estramustine (Cat: E0407) and Mitoxantrone (Cat: M6545), RPMI-1640 medium (Cat: R0883), L-glutamine (Cat: G3126), trypsin (Cat: T2600000), antibiotic-antimycotic solution (with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL) (Cat: A5955) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) (Cat: S0415) was purchased from Biochrom (Darmstadt, Germany). LPA and all drugs were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and further diluted in culture conditions. The final concentration of DMSO was less than 0.1% in the culture medium.

### *Cells and culture conditions*

PC3 cell lines, provided by Salih Sanlioglu (Akdeniz University, Antalya, Turkey), were cultured in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin at 37°C in a humidified 5% $CO_2$ atmosphere.

### *Experimental groups for drugs and LPA treatment*

PC3 cells were treated with increasing concentrations (0, 1, 5, 10, 15 µM) of LPA in order to determine its concentration-dependent proliferative effects. In these cells, DNA synthesis was explained as cell proliferation marker assessed by BrdU incorporation test.

Eight groups were created for the other experiments. In the control group, cells were incubated at 37°C for 24 hours (h) in the cell culture media. In the LPA group, cells were incubated with LPA at a final concentration of 10 µM for 24 h at 37°C. In the docetaxel (Doc) group, cells were treated with the final concentration of 10 nM of Doc for 24 h. In the estramustine (Est) group, cells were incubated with Est at a final concentration of 10 µM for 24 h. In the mitoxantrone (Mit) group, cells were incubated with Mit at a final concentration of 0.2 µM for 24 h. In the LPA plus Doc group, cells were treated with 10 µM of LPA and 10 nM of Doc for 24 h. In the LPA plus Est group, cells were incubated at 37°C for 24 h with 10 µM of LPA and 10 µM of Est. In the LPA plus Mit group, cells were incubated at 37°C for 24 h with 10 µM of LPA and 0.2 µM of Mit.

### *Cell proliferation assay*

Cell proliferation ELISA BrdU (5-Bromo-2'-deoxyuridine) colorimetric assay kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure cell proliferation. Manufacturer's instructions were followed. After BrdU was incorporated into newly synthesized DNA, its reaction product (antibody bound BrdU) was quantified by measuring the absorbance at 370 nm wavelength by using ELISA plate reader (Thermo Labsystems, Multiskan Spectrum, Waltham, MA, USA) in samples of at least three independent experiments.

### *Colony-formation assay*

Colony formation assay was performed by the method of Mossman T[18]. After LPA and/or drug treatments for 24 h, cells were trypsinized, counted, and seeded at the appropriate densities. Cultures were incubated for ≈ 12 days at 37°C until the colony formation can be visualized under the bright-field microscope. After the medium removed gently, absolute methanol was added for fixation of cells. Colonies were stained with 1% crystal violet after removing methanol, and finally rinsed with water. The stained colonies (at least 20 cells) were counted. At least three independent experiments were considered for calculating the mean values of colonies in each group.

### *Apoptosis detection*

Annexin V-FITC kit (Enzo Life Science International Inc, Plymouth Meeting, PA, USA) was used to detect apoptosis in the cells. Trypsinized cells were centrifuged at 300 x g for 5 min at 4°C. The cell pellet was stained with annexin-V FITC and PI as described in the Manufacturer's instructions. A minimum of 10,000 events per sample tube were acquired by using flow cytometer (BD FACS Canto II Flow Cytometer, BD Biosciences, San Jose, CA, USA). FACS Diva software (BD Biosciences, San Jose, CA, USA) was used for analyzing the data within 1 hour of staining. Each sample was run in triplicate.

### *Statistical Analysis*

Data was expressed as mean ± standard deviation (SD). The data was analyzed using statistical package program SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Student's *t* test and one-way ANOVA were used for comparison of the groups. We used Kruskal-Wallis test to compare samples from two or more groups in clonogenic experiments.

## RESULTS

### *Lysophosphatidic acid induced cell proliferation in PC3 cells*

We first evaluated the effect of different concentrations of LPA (0, 1, 5, 10, 15 μM) on the cell proliferation by measuring the level of BrdU incorporation into newly synthesized DNA. LPA was found to increase cell proliferation in a concentration-dependent manner (Figure 1). The maximum incorporation of the BrdU into the DNA of proliferating cells was observed in the presence of 10 μM of LPA.

As shown in Figure 2, all three drugs (Doc, Est, and Mit) significantly decreased cell proliferation compared to controls ($p< 0.001$). The treatment of cells with each drug in combination with LPA significantly augmented the percentage of viable cells compared to only drug-treated cells

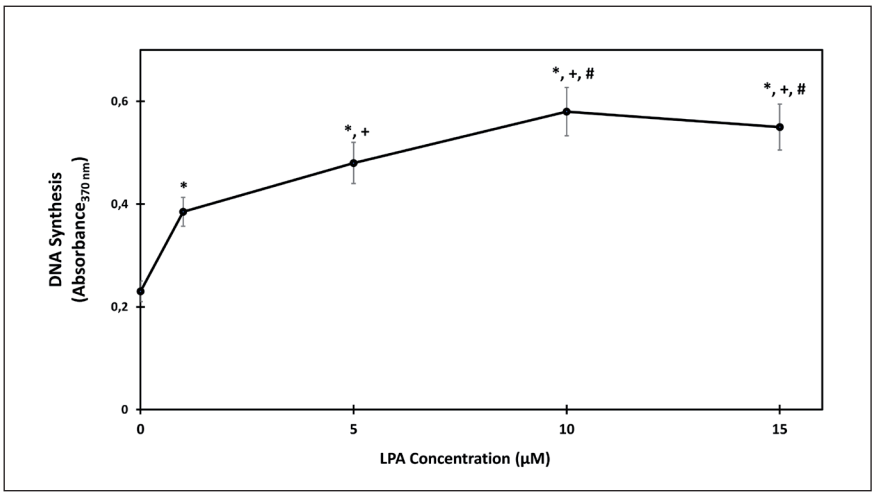


**Figure 1.** The effect of LPA concentrations on the cell proliferation in PC3 cells. Cells were treated with 0, 1, 5, 10 or 15 μM of LPA for 2 h. In order to explore cell proliferation, newly synthesized DNA levels were measured by using the BrdU incorporation assay kit. Data was expressed as mean ± SD of three independent experiments. *: $p<0.05$ compared to control (0 μM LPA), +: $p<0.05$ compared to 1 μM LPA, #: $p<0.05$ compared to 5 μM LPA.

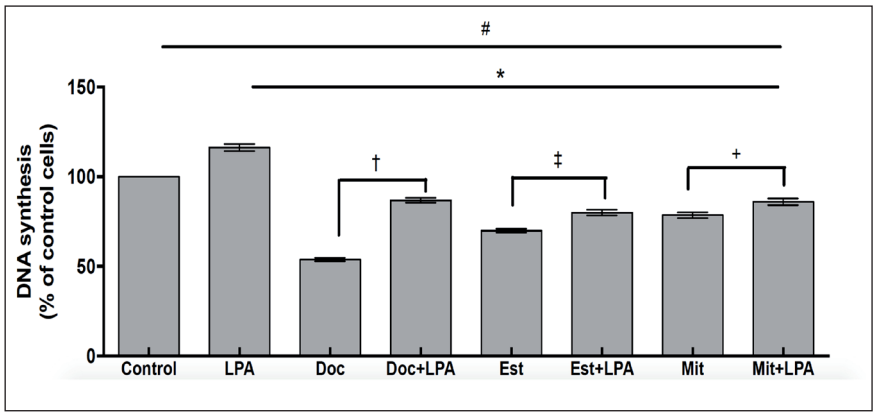


**Figure 2**. Effects of LPA and drugs on cell proliferation in PC-3 cells. PC3 cells were treated with LPA (10 µM), Doc (10 nM), Est (10 µM), and Mit (0.2 µM) for 24 h. Results were expressed as means ± SD of three independent experiments. #: $p<0.05$ all groups compared to control, *: $p<0.05$ all drug groups compared to LPA, †: $p<0.001$ Doc+LPA group compared to Doc, ‡: $p<0.01$ Est+LPA group compared to Est, +: $p<0.05$ Mit+LPA group compared to Mit.

($p<0.001$ for Doc+LPA *vs*. Doc; $p<0.05$ for Est+LPA *vs*. Est; $p<0.05$ for Mit+LPA *vs*. Mit). The lowest percentage of viable cells was found in Doc (10 nM) group. LPA was detected to be more effective in Doc + LPA group ($p<0.001$) among drug-treated cells for increasing cell proliferation (Figure 2).

### *Effects of LPA on colony formation in PC3 cells*

The effects of LPA and/or drugs on colony forming ability of PC3 cells were expressed as the percentage of colony forming cells in Figure 3. LPA increased colony formation in PC3 cells ($p<0.05$). We found that all drugs reduced the percentages of colonies, dramatically ($p<0.001$). Co-treatment of each drug with LPA significantly ($p<0.01$) increased the number of colonies as compared to the groups treated with drugs only.

### *Effects of LPA on apoptosis in PC3 cells*

Apoptotic index was revealed by Annexin V-FITC and PI staining as shown in representative dot plots (Figure 4A) and bar graphs (Figure 4B and 4C). There was no significant difference between control and LPA group in the percentages of apoptotic cells, while a slight increase was found in the percentage of viable cells of LPA group. The percentage of viable cells were significantly decreased in all drug-treated groups when compared to the control. However, when the cells were treated with drugs combined with LPA, there were significant increases in the percentage of viable cells. As shown in Figure 4C, the percentages of total apoptotic cells which were treated chemotherapeutic drugs, were found to be increased significantly compared to control ($p<0.001$). Co-treatments of each drug with LPA were found to significantly ($p<0.001$) reduce the percentages of total apoptotic cells.

## DISCUSSION

In this study, we investigated the effects of LPA against cell toxicity of docetaxel, estramustine, and mitoxantrone in PC3 cells. Initially, we have examined their effects on PC3 cell proliferation. All three agents were found to decrease the levels of PC3 cell proliferation. When we compared these three drugs by their cell death inducing properties, docetaxel was found to be the most effective agent. This effect may be due to its high microtubule polymerization capacity, being taken into the cell quickly or remaining within the cell for a long time[19].

LPA was found to increase cell proliferation in a concentration-dependent manner and the maximum concentration for incorporation of the BrdU into the DNA of proliferating cells was 10 µM. In our study, treatment of cells with LPA plus chemotherapeutic agents increased cell proliferation values compared to only drug-treated groups. There are some studies showing that cell proliferation in some cancer cells is induced by increased LPA[20,21]. One study revealed that LPA stimulates cell proliferation in PC3 human prostate cancer cells[22]. These findings are in accordance with our findings. We suggest that LPA may have a detractive role against cytotoxic effects of chemotherapeutic drugs and increase cell proliferation in PC3 cells.

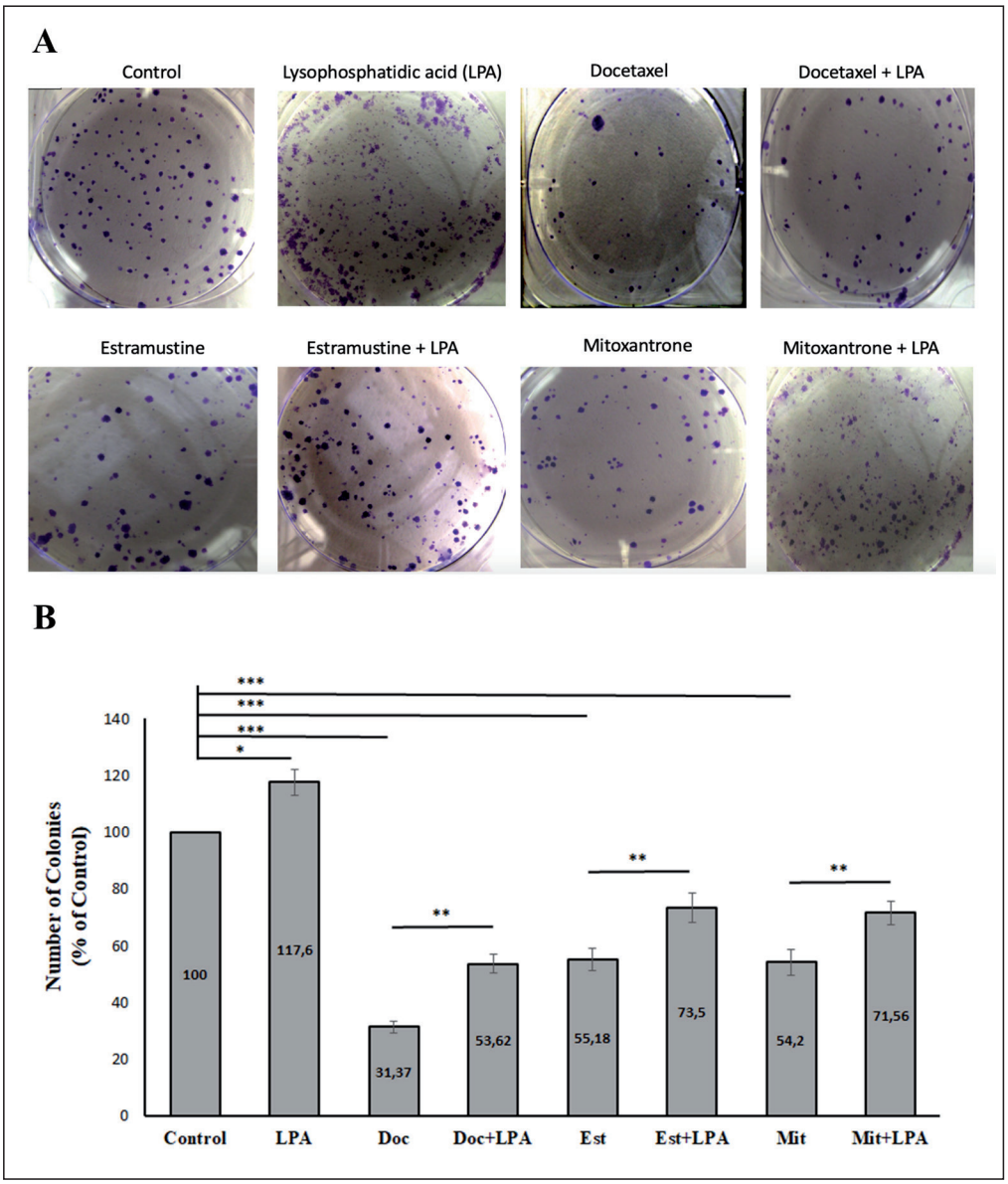


**Figure 3.** Effect of LPA (10 µM) on the ability of colony formation in PC-3 cells treated with or without chemotherapeutic drugs (Doc (10 nM), Est (10 µM), and Mit (0.2 µM)). Cells were incubated for 12 days at 37°C. The stained colonies were counted and colonies containing at least 20 cells were scored. Results were expressed as means ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

In our study, treatment of cells with LPA increased the number of colonies while three drugs (docetaxel, estramustine, and mitoxantrone) decreased compared to control group. Colony forming abilities of the cells were found to be increased in each drug-LPA-cotreated group. As compatible with our findings, Wang et al[23] and Jourquin et al[24] also reported that LPA increased the number of colonies in ovarian and epithelial cancer cells. In a study of Fukushima et al[25], they reported that knockdown of LPA1 and LPA3 receptors inhibited the colony formation of pancreatic cancer cells.

An effective chemotherapy is supposed to induce apoptosis. However, disruptions may occur in apoptotic signaling pathways due to the development of drug resistance and modifications in intracellular signals in cancer cells[26]. In the present study, we investigated the effects of LPA, chemotherapeutic drugs, and their combinations with LPA on the apoptotic index in PC3 cells. LPA was found to increase viable cells and to decrease the percentage of necrotic cells (Figure 4B). We examined the effect of LPA on the apoptotic index in PC3 cells treated with three chemotherapeutic drugs. We found that docetaxel, estramustine and mitoxantrone have augmented apoptotic cells whereas treatment of cells with LPA plus drugs decreased the apoptotic cells (Figure 4C). Docetaxel is known to induce apoptosis by means of phosphorylation of Bcl-2 protein and therefore

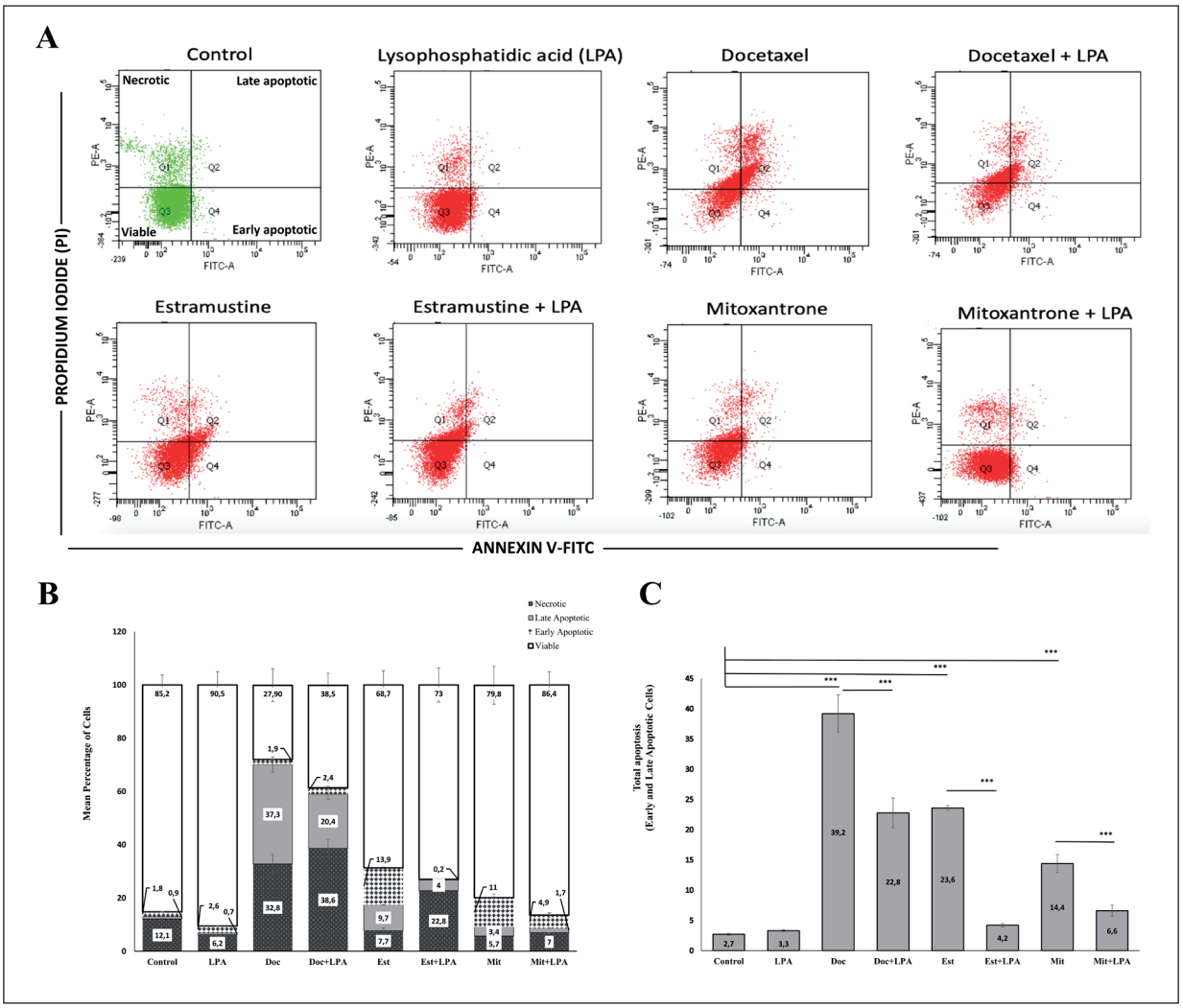


**Figure 4.** Effect of LPA (10 µM) on apoptosis in PC-3 cells treated with or without chemotherapeutic drugs (10 nM of Doc, 10 µM of Est and 0.2 µM of Mit). (A) Representative dot plots show the distribution of apoptotic, necrotic and viable cell populations which were stained with PI (vertical axis) and annexin-V conjugated to FITC (horizontal axis). Q1 ($PI^+$/Annexin $V^-$) shows necrotic cells; Q2 ($PI^+$/Annexin $V^+$) shows late apoptotic cells; Q3 ($PI^-$/Annexin $V^-$) shows viable cells; Q4 ($PI^-$/Annexin $V^+$) shows early apoptotic cells. (B) Bar graph representing the percentages of viable, early apoptotic, late apoptotic and necrotic cells as means ±SD of triplicate samples. (C) Bar graph representing the percentage of total apoptotic cells (early and late) as means ± SD of triplicate samples. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

inhibition of anti-apoptotic activity of Bcl-2[27]. Moreover, LPA has been reported to activate ERK signaling and to promote tumor cell migration[28]. The mechanism of decrease in apoptotic effect of docetaxel by LPA may be due to the upregulation of Bcl-2 via the ERK signaling pathways activated by LPA. Estramustine induces apoptosis by inhibiting microtubule function and increasing phosphorylation of Bcl-2[29]. In a previous study, it has been shown that one of the apoptotic mechanisms of estramustine is the up-regulation of proapoptotic Bak (Bcl-2 homologous antagonist/killer, belongs to Bcl-2 protein family) expression[30]. The potent apoptotic effect of this drug may be due to its capability to induce the expression of Bak protein. We found that LPA significantly inhibited apoptosis in estramustine-treated PC3 cells (Figure 4). There are several studies showing the potential effect of LPA on cell proliferation, survival and migration[31,32]. In accordance with our study, it was proposed new therapeutic models targeting LPA, its receptors and signaling pathways[33,34].

## CONCLUSIONS

As a result, our study reveals the effect of LPA on the cytotoxicity of three chemotherapeutic drugs (docetaxel, estramustine, and mitoxantrone) in PC3 prostate cancer cells. We suggest that any strategy suppressing the effects of LPA may be a useful approach in cases of chemotherapy of

prostate cancer. The usage of LPA inhibitors may be a promising approach in order to improve the chemotherapeutic potential of the drugs.

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**Conflict of interest:**
The Authors declare that they have no conflict of interests.

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