

## Original Article

**Running Title:** Combined Effect of Sodium Valproate and Lithium Chloride on Apoptosis of PC3 Cells

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### Combined Effects of Sodium Valproate and Lithium Chloride on Inducing Apoptosis and Inhibiting Invasion of PC3 Cells

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#### Abstract

**Background:** In this study, we aimed to investigate the combined effect of sodium valproate (SV) and lithium (Li) against viability, migration, and invasion of prostate cancer cell line.

**Method:** In this in vitro study, PC3 cells were treated with different concentrations of SV (2.5, 5.0, and 10 mM) and Li (2.5, 5.0, and 10  $\mu$ M) either alone or in combination in this in vitro investigation. Using the MTT test and annexin V/7ADD flow-cytometry, cell viability and apoptosis were assessed. Transwell chamber test was used to assess PC3 cells' invasion and migration.

**Results:** SV and Li alone had no significant on PC3 cell viability. However, the combination of SV and Li in all tested concentrations decreased the viability of PC3 cells in a dose dependent manner ( $P < 0.001$ ). The combination of SV and Li (5.0  $\mu$ M + 5.0 mM) increased apoptosis of PC3 cells compared to the control group ( $P = 0.003$ ). Transwell assay showed that combination of SV with Li (5.0  $\mu$ M + 5.0 mM) reduced the migration and invasion PC3 cells significantly. The lack of a significant difference between the predicted and observed fractional inhibition for the effects of SV+Li suggests that SV and Li may have synergistic effects on lowering PC3 cell viability and invasiveness.

**Conclusion:** This study's findings show that combined low doses of SV and Li could decrease the viability and invasiveness of the PC3 cells; therefore, it can be considered as a new strategy for the treatment of PCa. Furthermore, in vivo studies are required to confirm the results of this study.

**Keywords:** Prostatic neoplasms, Valproic acid, Lithium, Combined modality therapy, Apoptosis

## **Introduction**

With over one million new patients a year and about 400000 death cases, prostate cancer (PCa) is the most common as well as aggressive urogenital malignancy among men globally.<sup>1</sup> Despite the advantages of standard treatments such as radical prostatectomy, radiation, and chemotherapy, metastasis to distant organs and drug resistance<sup>2</sup> pose a danger to the patients' quality of life.<sup>3</sup> Thus, using safer and more effective strategies, with the ability to target PCa tumor cell proliferation and metastasis, is the focus of many efforts. PCa is known as a complex disease with defects in several signaling pathways.<sup>4</sup> Therefore, combination therapy that uses two or more drugs against different molecular pathways has emerged as a promising modality in PCa therapy.<sup>5, 6</sup>

Histone deacetylases inhibitors (HDACi) and glycogen synthase kinase-3beta inhibitors (GSK-3BI) are amongst the drugs widely used in the combination therapy of cancer. Many tumor cell proliferation and metastasis-inhibiting medicines, including HDACi, have been proposed.<sup>7</sup> The effectiveness of HDACi in preventing tumor angiogenesis and the epithelial to mesenchymal transition has also been shown.<sup>7,8</sup> HDAC upregulation in association with poor prognosis was reported in patients with PCa.<sup>9</sup> In addition, it was reported that HDACi decreased proliferation of prostate tumor cells through inhibiting the expression and activity of telomerase.<sup>10</sup> Qian et al. reported that belinostat, a HDACi, potently inhibited tumor cell proliferation through inducing G2/M arrest. Furthermore, belinostat inhibited the migration of prostate tumor cells and increased the production of tissue inhibitor of metalloproteinase-1 (TIMP-1) by these cells. Belinostat increased the expression of p21 and decreased the expression of potentially oncogenic proteins (mutant p53 and ERG).<sup>11</sup> GSK-3B is a serine/threonine kinase with a wide range of

physiological activity, including the control of cell growth and differentiation. Besides, the role of GSK-3beta in several pathological conditions, notably tumorigenesis, has been implicated. GSK-3 upregulation in PCa was shown in the patients with the disease. Furthermore, research using animal models has shown that GSK-3B inhibitors (GSK-3BI), such as lithium (Li), have an inhibitory impact on tumor growth and metastasis.

Regarding the important role of HDAC and GSK-3B in the pathogenesis of most of cancers and the relative safety of HDACi and GSK-3BI, the synergistic/additive effect of combined HDACi and GSK-3BI in the suppression of tumor cell growth and metastatic properties were evaluated on several tumor cell lines and animal models.<sup>13,14</sup> We evaluated the effects of combination of sodium valproate (SV), a well-known HDACi,<sup>15</sup> and Li on the cell viability as well as metastatic characteristics of PCa cells. To do this, the PC3 cell line was exposed to various concentrations of SV and Li, either alone or together, and the cells' survival, apoptosis, migration, and invasion were assessed using the proper techniques.

## **Material and Methods**

### ***Cell culture conditions***

In this in vitro study, PC3 was purchased from Pasture National Cell Bank (Tehran-Iran). The cells were cultured in RPMI 1,640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen Corporation), penicillin (100 IU/ml) and streptomycin (100 mg/ml) and maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Stock solution of SV (Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The final concentration of DMSO in the culture medium of all experimental groups, including the control group was 0.1% which had no significant effects on cell viability.

### ***Cell viability assay***

MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was used to evaluate the effect of the treatments on PC3 cell viability. Briefly,  $1 \times 10^4$  of PC3 cells were seeded per well in 96 well plates. After 24 hours, the cells were treated with various concentrations of SV (2.5, 5.0, and 10.0  $\mu$ M) and LiCl (2.4, 5.0, and 10.0 mM) alone or in combination for 48 hr. MTT assay was performed by addition of 20  $\mu$ l of MTT solution (5mg/  $\mu$ l in PBS) to the culture medium and incubated at 37°C for 4 hr. 150  $\mu$ l of DMSO was added into each well to solubilize the formed formazan. After 10 minutes of shaking, the absorbance of each well was measured at 490nm wavelength using ELISA reader.<sup>16</sup>

### ***Annexin V/ 7-AAD apoptosis assay***

We used flow cytometric studies with annexin V/ 7-AAD (BD Pharmingen) to look into the potential impact of the compounds on PC3 cell apoptosis. In a nutshell, PC3 cells were grown in T25 culture flasks and exposed to SV (5.0  $\mu$ M) and Li (5.0 mM) either alone or together for 48 hours. The cells were then washed in cold phosphate buffered saline (PBS) and suspended in 1 ml of ice-cold binding buffer. Annexin V-PE (5  $\mu$ l) and 7-AAD (5  $\mu$ l) were then added to 100  $\mu$ l of cell suspension, gently mixed, and incubated for 15 min in darkness at room temperature. After 15 minutes, 400  $\mu$ l of 1  $\times$  binding buffer was added, and the apoptotic cells were analyzed using BD FACSCalibur (Becton Dickinson, USA). A total of 10000 events were obtained for each sample. The percentage of total apoptosis (early + late apoptotic cell) was calculated for each sample.

### ***Invasion and migration assay***

Transwell migration and invasion assays were performed to investigate the effects of the drugs on invasiveness properties of PC3 cells. For the migration assay, the cells were serum starved for an overnight. The lower

part of the transwell chamber (8 mm pore size, BD Bioscience, Bedford, MA) was filled with RPMI 1640 with 10% FBS, and  $5 \times 10^4$  of the treated as well as control PC3 cells were suspended in serum-free medium and added to the upper part of the transwell chamber. After incubation for 24 hours in 37°C, the migrated cells were fixed in methanol, stained in 0.1% crystal violet, and counted in 5 fields of inverted microscope (200 $\times$  magnification). The invasion assay was carried out in a similar manner, except that the cells were seeded into transwell coated with 100 ml of 1:2 Matrigel (Matrigel, Corning incorporated, USA) and incubated for 48 hours.

### ***Statistical analysis***

At least five independent timings were included into each experiment. For statistical analysis, SPSS16.0 software (SPSS, Chicago, IL, USA) was utilized. The results were analyzed using the student t-test and one-way ANOVA. The statistically significant difference between the groups was defined as  $P < 0.05$ . Data were expressed as mean  $\pm$  standard deviation (SD). The possible interaction (synergistic or additive) among the treatments (SV and Li) was evaluated using Bliss equation<sup>17</sup> ( $E_{bliss} = EA + EB - (EA \times EB)$ ), where EA = fractional inhibition (FI) obtained by SV, EB = FI obtained by Li, and  $E_{bliss}$  = expected FI obtained by the combination of SV and Li (SV+Li). The values of  $E_{bliss}$  and experimentally measured FI (observed FI) for each combination were compared using Student t-test to determine the type of interaction. The percentage of FI was calculated using the following formula, %FI = [(absorbance of control – absorbance of test)/absorbance of control]  $\times$  100. If no significant difference was observed between  $E_{bliss}$  and EFI values, the interaction was considered additive and if  $E_{bliss}$  value was significantly lower than EFI, the interaction was defined as synergistic.

## Results

### *The effects of combined SV and Li treatment on the viability of PC3 cells*

The impact of SV and Li, either alone or in combination, on the viability of PC3 cells was assessed using the MTT test. Figure 1A demonstrates that 48 hours of treatment with SV (2.5 and 5.0 M) had no appreciable influence on the viability of PC3 cells. However, SV at 10  $\mu$ M concentration caused a significant decrease in the cell viability compared to the control group ( $P < 0.001$ ). The same results were obtained for Li where 2.5- and 5mM concentration of Li had no significant effects on cell viability, while 10 mM concentration of Li caused a significant decrease in PC3 cells viability ( $P = 0.003$ ) (Figure 1B). The combination of SV and Li in all tested concentrations (2.5 + 2.5, 5.0 + 5.0 and 10.0 + 10.0) decreased the viability of PC3 cells compared to the control group in a dose dependent manner. The anticipated and observed FI values for the combined effects of SV and Li were not substantially different at the three doses tested, as shown in table 1, indicating an additive impact of SV and Li on reducing PC3 cell viability.

### *The effects of SV and Li treatment, alone or in combination, on apoptosis of PC3 cells*

Given the reducing impact of SV and Li combination on cell viability, the effects of SV and Li on apoptosis of the PC3 cells were investigated, using annexin V/ 7-AAD flowcytometry. The data revealed that SV at 5.0  $\mu$ M concentration caused a significant increase in the percentage of apoptotic cells compared to the control cells, while 5 mM of Li had no significant effects on apoptosis of PC3 cells (Figure 3). Additionally, the results showed a significantly higher rate of apoptosis in PC3 cells treated with SV (5.0 M) and Li (5.0 mM) compared to the control group ( $P = 0.003$ ). The expected value for the combined effect of SV and Li (5.0 $\mu$ M of SV+5.0 mM f Li) on decreasing cell viability of PC3 cells in apoptosis test ( $37.8 \pm 19.4\%$ )

was not significantly different from the experimental value ( $40.8 \pm 7.5 \%$ ) ( $P = 0.858$ ), suggesting an additive effect of SV and Li on inducing apoptosis of the PC3 cells.

### *The effects of SV and Li treatment on the migration and invasion of PC3 cells*

The effects of SV and Li on cell migration and invasion of PC3 cells were evaluated using transwell assay. As shown in figure 4A, SV and Li significantly decreased the number of migrated cells, as compared to the control group ( $P < 0.001$ ). Furthermore, the combination of SV with Li reduced the migration of the PC3 cells (Figure 4A) significantly ( $P < 0.001$ ). The expected FI for the effects of SV+Li on migration was calculated to be  $42.34 \pm 14.9\%$  which was not significantly different from the experimental FI ( $37.85 \pm 10.32\%$ ) ( $P = 0.184$ ), suggesting an additive effect of SV and Li on reducing the cell migration ability of the PC3 cells. Both SV and Li substantially reduced the number of invading cells in the PC3 cells when compared to the control group ( $P = 0.033$  and  $P = 0.022$ , respectively) (Figure 4B). Furthermore, combination of SV with Li reduced the PC3 cells invasion (Figure 4B) significantly ( $P < 0.001$ ). Expected FI for the combination of SV and Li (Ebliss =  $38.4 \pm 16.02\%$ ) was not significantly different from the experimental value (EFI =  $41.28 \pm 14.9\%$ ,  $P = 0.478$ ), suggesting an additive effect among SV and Li on reducing the cell invasion ability of the PC3 cells.

## Discussion

The possible synergistic/additive effects of SV and Li cotreatment on the viability and invasiveness of the PC3 cells were evaluated. The findings showed the dose-dependent significant effects of SV and Li to decrease the cell viability, inducing apoptosis, and inhibiting migration and invasion of PC3 cells. Additionally, the findings of combination therapy showed a substantial additive impact of modest doses of SV and Li

on the invasiveness and cell survival of PC3 cells, indicating the potential effectiveness of this combination in the treatment of PCa.

In the last two decades, using small molecules that target signal transduction pathways such as tyrosine kinase inhibitors, HDACs, GSK3BIs, and mTOR inhibitors was the focus of cancer targeted therapy. Nevertheless, toxicity for the normal cells and drug resistance have limited their use.<sup>18</sup> Combination treatment is an efficient way to get over these restrictions due to its benefits over monotherapy, which include higher effectiveness, fewer side effects, and the avoidance of drug resistance.<sup>19</sup> The potency of SV<sup>20</sup> and Li<sup>21</sup> to inhibit proliferation and inducing apoptosis of many tumor cells including androgen dependent and androgen independent of PCa tumor cells was shown. The synergistic/additive effects of HDACs with some several cytotoxic compounds were revealed in previous studies. Recent research by Edderkaoui et al. has shown that pancreatic ductal adenocarcinoma cells exhibit reduced invasiveness and cause death when HDACs and GSK3B are simultaneously inhibited by metaver, a dual inhibitor of both enzymes. The safety and efficacy of combined Li and SV against glioblastoma (GBM) was revealed in a previous studies using animal models and patients with GBM. It was demonstrated that a cocktail containing SV, Li cimetidine, and olanzapine could significantly inhibit cell invasion and proliferation of GBM cells and increased survival of patients with GBM.<sup>22</sup> Additionally, a prior research found that the down-regulation of proteins involved in the homologous recombination pathway of DNA repair had a synergistic impact between PARP inhibitors and HDACi in reducing cell viability and clonogenicity as well as enhancing apoptosis of the PCa cell. Several earlier investigations have also shown the synergistic effects of Li with cytotoxic medicines in reducing PCa cell viability.

Erguen et al. showed that cotreatment of Li with cytotoxic drugs, including doxorubicin, etoposide, and vinblastine decreased the viability of PCa cell lines in a synergistic manner.<sup>23</sup> The same results reported by Azimian et al. revealed a significant synergistic effect between Li and cytotoxic agents in decreasing cell viability and inhibiting the cell cycle of LNCaP cells.<sup>24</sup> In this study, our data showed that a combination of the low dose of SV and Li was more potent than either drug alone in decreasing the viability of PC3 cells. Apoptosis plays a critical role in cancer pathogenesis and treatment.<sup>25</sup> The common characteristic of tumor cells is reduced cell apoptosis caused by dysregulation of the molecules involved in apoptotic pathways, such as increased pro-apoptotic proteins, decreased anti-apoptotic proteins, and alteration in caspase activity. On the other hand, medications that may activate apoptotic pathways can kill tumor cells and are likely a worthwhile target for cancer treatment.<sup>25</sup> Since our data revealed a significant decrease in the cell viability after the PC3 cells treatment with SV or Li, apoptosis assay was carried out to identify the role of apoptosis in SV- and Li-induced cell death. Findings of this study revealed that while SV (5.0  $\mu$ M) or Li (5mM) had little or no significant effects on PC3 cells apoptosis, treatment with the combination of SV (5.0  $\mu$ M) and Li (5.0 mM) increased the percentage of apoptotic cells compared to the control group, suggesting an additive effect among SV and Li on inducing the apoptosis of the PC3 cells. According to reports, Li caused tumor cells to undergo apoptosis via P53-independent processes, Bcl-2 downregulation, and modification of Fas-dependent signaling pathways.<sup>26</sup> The apoptotic effects of SV have been attributed to the same mechanism.<sup>27</sup> Our data clearly show that combined low doses of SV and Li enhance the apoptosis and decrease the

viability of PC3 cell, a null P53, invasive, and androgen- insensitive PCa cell line.<sup>28</sup> The use of SV and Li in combination is advised for the treatment of PCa since loss of p53 is often seen in PCa and is linked to a poor prognosis.<sup>29</sup> To ascertain the advantages of this combination in the PCa therapy, further research is necessary.

Metastasis to distant organs commonly occurs in PCa. Thus, we evaluated the effect of SV and Li, alone or in combination, on invasion and migration abilities of the PC3 cells. In accordance with the results of previous investigation, the findings of this study show that the use of both SV and Li per se is able to reduce the invasion and migration of the PC3 cells. Furthermore, our research showed that the addition of SV and Li reduces the invasiveness of PC3 cells. The metastatic spread of PCa tumor cells involves matrix metalloprotease-2 and -9.<sup>30</sup> Our research revealed that the combination of SV and Li had no appreciable impact on the mRNA expression of MMPs (data not shown). We did not evaluate the effects of the drugs on MMPs expression at protein level and MMPs activity. Furthermore, the activity of MMPs is influenced by tissue inhibitors of MMPs.<sup>31</sup> Thus, more studies are needed to determine the role of MMP-2 and -9 to inhibit the effects of SV and Li on migration and invasion of the PC3 cells.

Angiogenesis plays an important role in PCa, so anti-angiogenesis therapy has emerged as a promised method in PCa therapy.<sup>32</sup> Recently, Afzal et al.<sup>33</sup> evaluated the effect of combination of Li and SV on angiogenesis using chorioallantoic membranes (CAMs) model. They demonstrated that the combination of Li and SV reduced the number of vascular branch points, which is a measure of angiogenesis. This suggests that the two therapies may have combined inhibitory effects on cancer angiogenesis. To learn more about the potential positive effects

of Li and SV combination on PCa angiogenesis, further research is required.

We used a combination of reliable methods including MTT assay, apoptosis assay, migration, and invasion assay to show beneficial combined effects of SV and Li on viability and invasiveness of prostate cancer cell line. The key drawback of the present study is the use of in vitro research without consideration of the in vivo effects of the medications in PCa animal models. Additionally, the current research did not look into potential molecular pathways that could mediate the combined effects of SV and Li.

### **Conclusion**

All in all, this study's findings show that combined low doses of SV and Li could decrease the viability and invasiveness properties of the PC3 cells; therefore, it can be considered a new strategy for the treatment of PCa. Nevertheless, our data are preliminary and further studies particularly on animal models of PCa are required to confirm the results of the present study.

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### **Conflict of Interest**

The authors declare that they have no conflicts of interest.

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Table 1. Comparison between expected and observed fractional index of cell viability inhibition

<b>SV(<math>\mu</math>M) + Li(mM)</b>	<b>Expected FI (%)</b>	<b>Observed FI (%)</b>	<b><i>P</i> values</b>
<b>2.5 + 2.5</b>	12.3 $\pm$ 11.0	15.5 $\pm$ 10.5	0.544
<b>5.0 + 5.0</b>	20.18 $\pm$ 18.0	17.4 $\pm$ 9.0	0.676
<b>10.0 + 10.0</b>	34.38 $\pm$ 12.3	31.31 $\pm$ 6.0	0.450

FI: Fractional index; SV: Sodium valproate; Li: Lithium. Expected FI values were calculated using Ebliss equation. The presented data are mean  $\pm$  S.D. of at least 10 independent experiments. Comparison between expected and observed values was done using Student T-test.

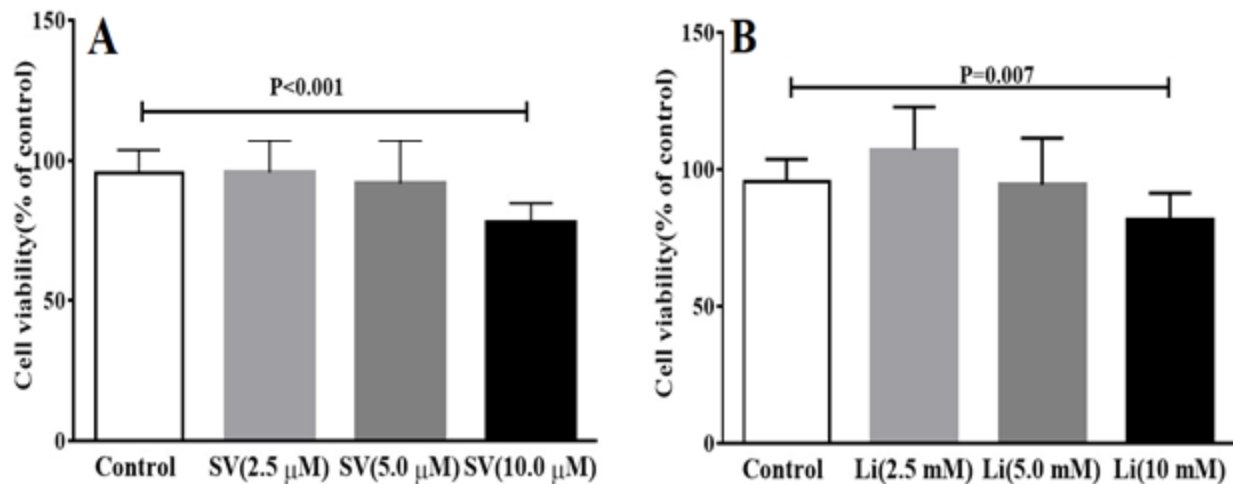


Figure 1. SV and Li reduced the viability of PC3 cell. PC3 cells were treated with SV (2.5, 5.0, and 10 μM) (A), Li (2.5, 5.0, and 10 mM) (B) and cell viability was evaluated, using MTT assay. Treatment with 2.5 and 5.0 μM of SV and 2.5 and 5.0 mM of Li alone had no significant effects on the viability of PC3 cells. The presented data are mean ± S.D. of at least 10 independent experiments which were analyzed using one-way ANOVA followed by LSD post hoc test. SV: Sodium valproate; Li: Lithium; MTT: [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

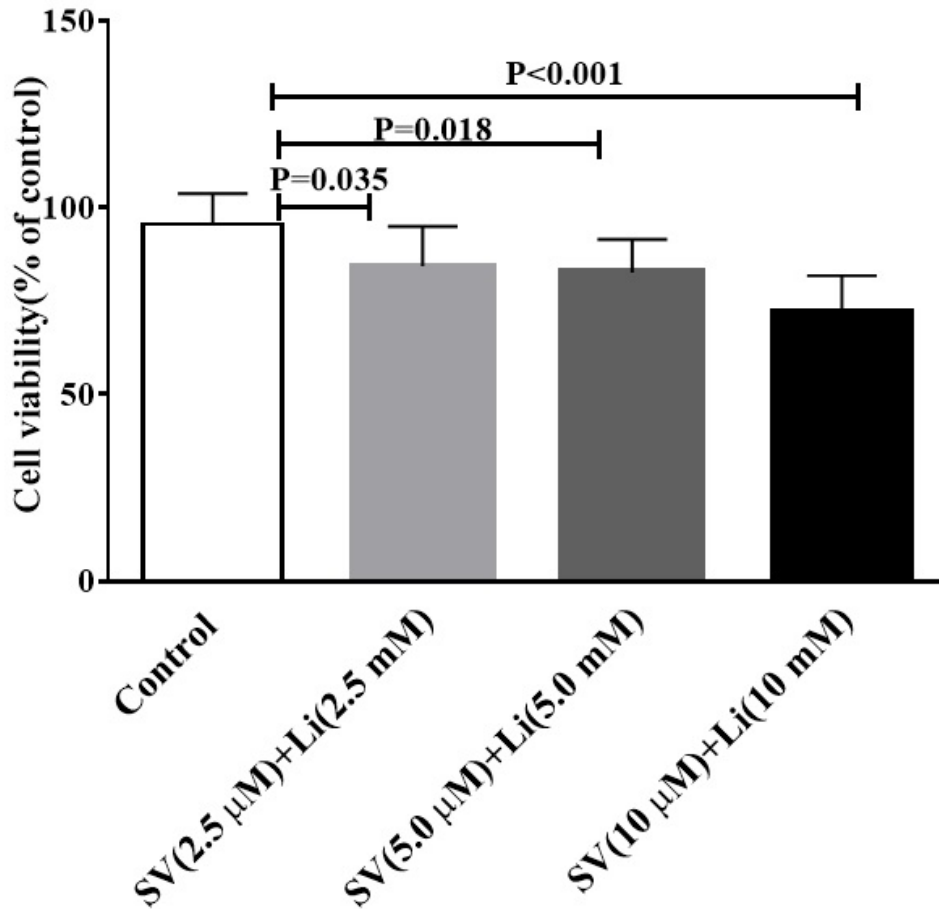


Figure 2. The combination of sodium valproate (SV) and lithium (Li) reduced the viability of PC3 cells in an additive manner. PC3 cells were treated with the combination of SV and Li, and cell viability was evaluated, using MTT assay. The data are mean  $\pm$  S.D. of at least 10 independent experiments which were analyzed using one-way ANOVA followed by LSD post hoc test. SV: Sodium valproate; Li: Lithium; MTT: [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

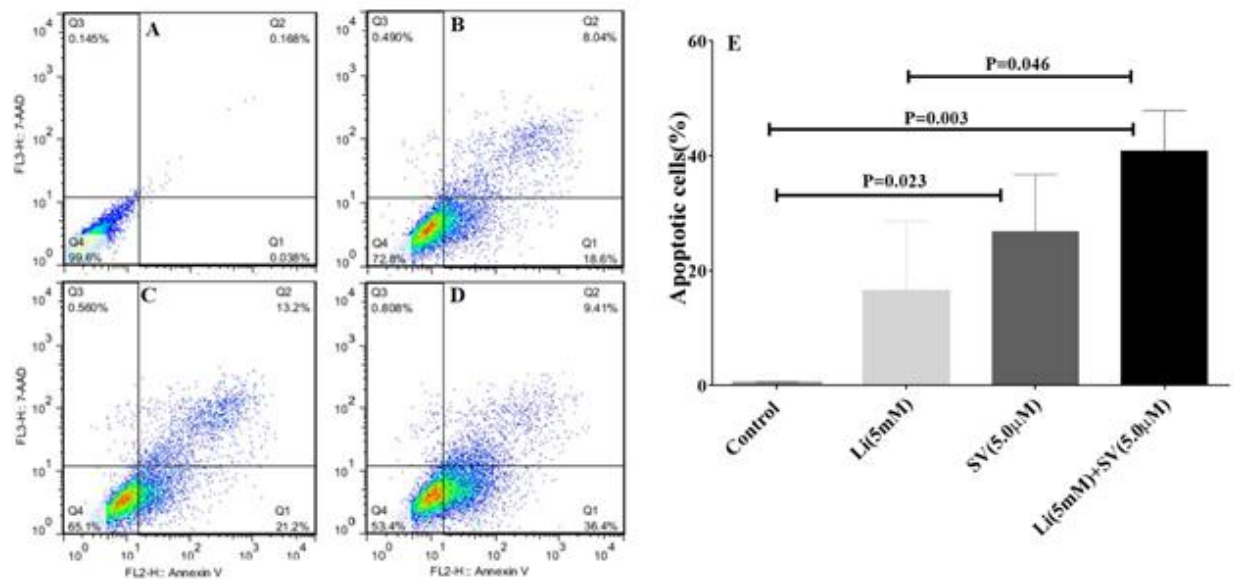


Figure 3. The combination of SV and Li increased the apoptosis of the PC3 cells. The flow cytometric charts show the percentage of early apoptotic (Q1), late apoptotic (Q2), dead cells (Q3), and live cells (Q4) in the control (A), Li (5.0 mM) (B), SV (5.0 μM) (C), and SV + Li (5.0 μM+5.0mM) (D) treated cells. The histogram shows the percentage of apoptotic cells (mean ± S.D) in various groups. The presented data are the mean ± SD of at least 3 independent experiments which were analyzed using one-way ANOVA followed by LSD post hoc test. SV: Sodium valproate; Li: Lithium

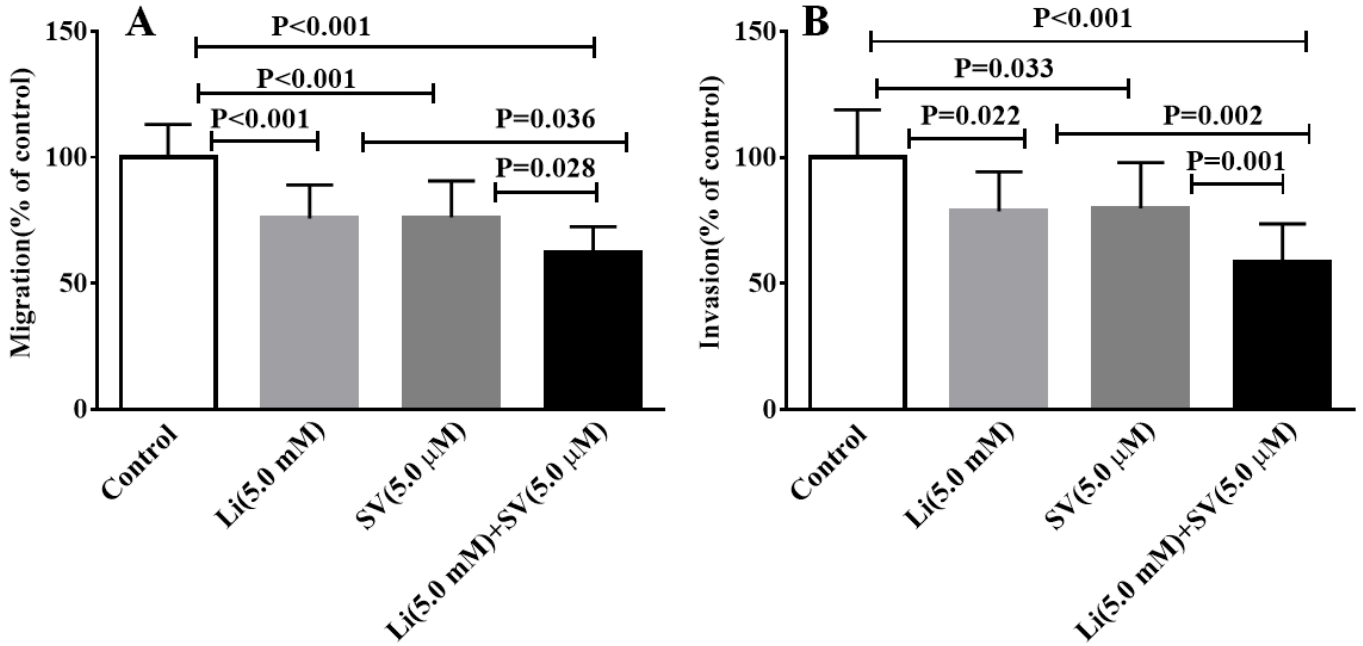


Figure 4. The combination of sodium valproate (SV) and lithium (Li) reduced migration (A) and invasion (B) of the PC3 cells. The data were analyzed, using Kruskal-Wallis test and presented as mean  $\pm$  S.D.

SV: Sodium valproate; Li: Lithium