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

Mohammad Ali Takhshid*, **♦, PhD, Samaneh Davoudzadeh*, PharmD, Ghazal Noohi***, PharmD, Samaneh Naderi*, MSc, Bahareh Zamani*, MSc**

*Division of Medical Biotechnology, Department of Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
**Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
***School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

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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 µM) and Li (2.5, 5.0, and 10 mM) either alone or in combination. 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 effects 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 µM + 5.0 mM) increased apoptosis of PC3 cells compared with the control group ($P = 0.003$). Transwell assay showed that combination of SV with Li (5.0 µM + 5.0 mM) reduced the migration and invasion of 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 additive effects on lowering PC3 cell viability and invasiveness.

Conclusion: These 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 prostate cancer. However, further in vivo studies are required to confirm the results of this study.

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

♦Corresponding Author:
Mohammad Ali Takhshid, PhD
Division of Medical Biotechnology, Department of Laboratory Sciences, School of Paramedical Sciences, Shiraz, Iran
Tel: +987132270238-40
Fax: +987132289113
Email: takhshidma@sums.ac.ir

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Introduction

With over one million new patients a year and about 400,000 death cases, prostate cancer (PCa) is the most common as well as aggressive urogenital malignancy among men globally.[1] Despite the advantages of standard treatments such as radical prostatectomy, radiation, and chemotherapy, metastasis to distant organs and drug resistance[2] pose a danger to the patients' quality of life.[3] 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.[4] Therefore, combination therapy that uses two or more drugs against different molecular pathways has emerged as a promising modality in PCa therapy.[5, 6]

Histone deacetylases inhibitors (HDACIs) 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 HDACIs, have been proposed.[7] The effectiveness of HDACIs in preventing tumor angiogenesis and the epithelial to mesenchymal transition has also been shown.[7,8] HDAC upregulation in association with poor prognosis was reported in patients with PCa.[9] In addition, it was reported that HDACIs decreased proliferation of prostate tumor cells through inhibiting the expression and activity of telomerase.[10] 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).[11] 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

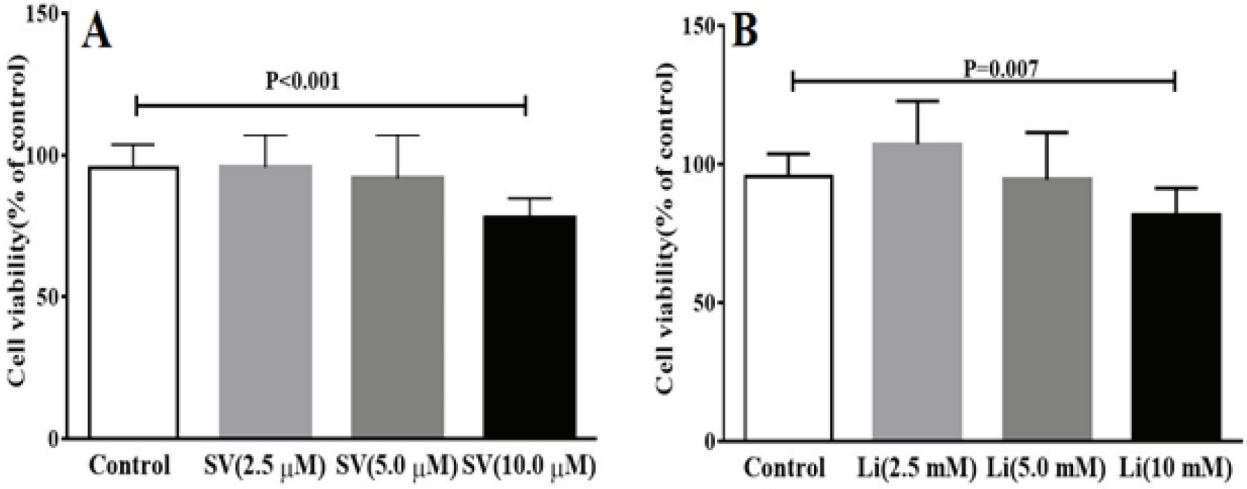

Figure1. SV and Li reduced the viability of PC3 cell. PC3 cells were treated with SV (2.5, 5.0, and 10 μM) (A), Li (2.5, 5.0, and 10 mM) (B) and cell viability was evaluated, using MTT assay. Treatment with 2.5 and 5.0 μM of SV and 2.5 and 5.0 mM of Li alone had no significant effects on the viability 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]

and the relative safety of HDACIs and GSK-3BI, the synergistic/additive effect of combined HDACIs and GSK-3BI in the suppression of tumor cell growth and metastatic properties were evaluated on several tumor cell lines and animal models.[13,14] We evaluated the effects of combination of sodium valproate (SV), a well-known HDACI,[15] 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 37ºC in a humidified 5% CO_2 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, 1×10^4 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 µM) 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 µl of MTT solution (5mg/ µl in PBS) to the culture medium and incubated at 37 ºC for 4 hr. 150 µl 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 490 nm wavelength using ELISA reader.[16]

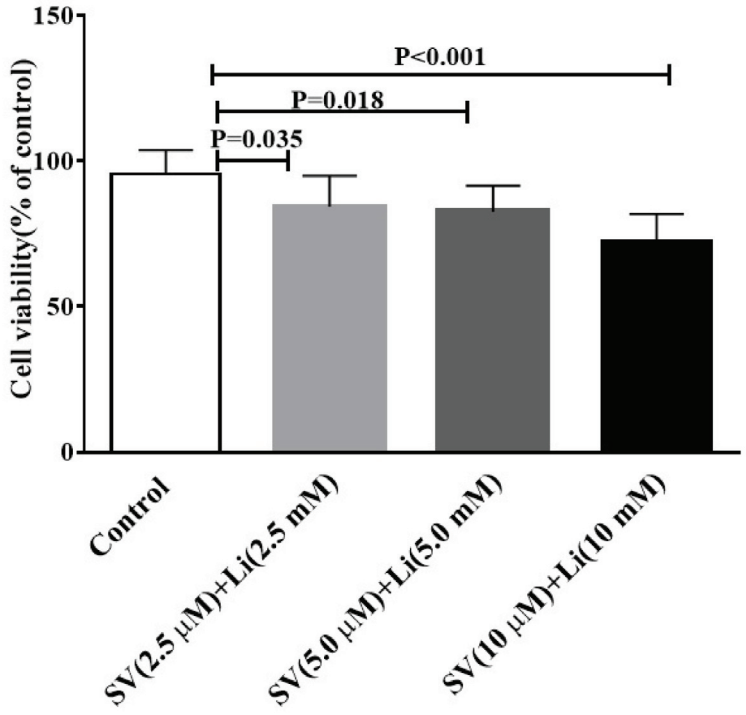

Figure 2. The combination of sodium valproate (SV) and lithium (Li) reduced the viability of PC3 cells in an additive manner. PC3 cells were treated with the combination of SV and Li, and cell viability was evaluated, using MTT assay. The data are mean ± S.D. of at least 10 independent experiments which 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]

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. The PC3 cells were grown in T25 culture flasks and exposed to SV (5.0 µM) 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 µl) and 7-AAD (5 µl) were then added to 100µl of cell suspension, gently mixed, and incubated for 15 min in darkness at room temperature. After 15 minutes, 400 ml of 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 the 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 5×10^4 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 37ºC, the migrated cells were fixed in methanol, stained in 0.1% crystal violet, and counted in 5 fields of inverted microscope (200× 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 ± standard deviation (SD). The possible interaction (synergistic or additive) among the treatments (SV and Li) was evaluated

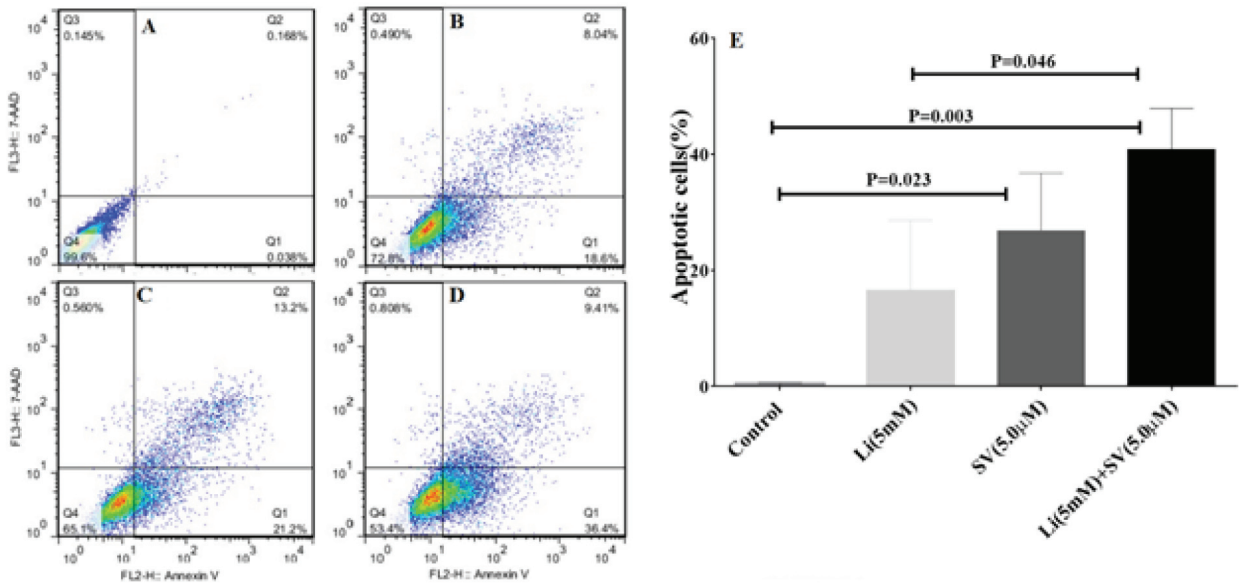

Figure 3. The combination of SV and Li increased the apoptosis of the PC3 cells. The flow cytometric charts show the percentage of early apoptotic (Q1), late apoptotic (Q2), dead cells (Q3), and live cells (Q4) in the control (A), Li (5.0 mM) (B), SV (5.0 µM) (C), and SV + Li (5.0 µM+5.0mM) (D) treated 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

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

SV(µM) + Li(mM)	Expected FI (%)	Observed FI (%)	*P* values
2.5 + 2.5	12.3 ± 11.0	15.5 ± 10.5	0.544
5.0 + 5.0	20.18 ± 18.0	17.4 ± 9.0	0.676
10.0 + 10.0	34.38 ± 12.3	31.31 ± 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 ± S.D. of at least 10 independent experiments. Comparison between expected and observed values was done using Student T-test.

using Bliss equation[17] (Ebliss = EA + EB (EA×EB), where EA = fractional inhibition (FI) obtained by SV, EB = FI obtained by Li, and Ebliss = expected FI obtained by the combination of SV and Li (SV+Li). The values of Ebliss 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] ×100. If no significant difference was observed between Ebliss and EFI values, the interaction was considered additive, and if Ebliss 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 µM) had no appreciable influence on the viability of PC3 cells. However, SV at 10 µM concentration caused a significant decrease in the cell viability compared with 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 with 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 (Figure 2).

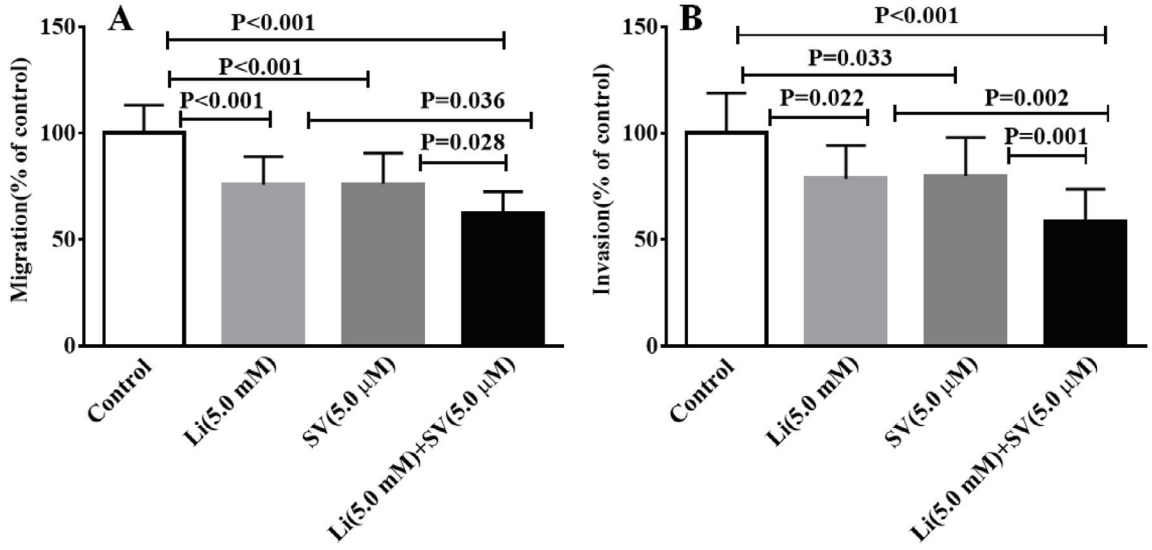

Figure 4. The combination of SV and Li reduced migration (A) and invasion (B) of the PC3 cells. The data were analyzed, using Kruskal-Wallis test and presented as mean ± S.D.
SV: Sodium valproate; Li: Lithium

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 flow-cytometry. The data revealed that SV at 5.0 µM concentration caused a significant increase in the percentage of apoptotic cells compared with 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 µM) and Li (5.0 µM) compared with the control group ($P = 0.003$).The expected value for the combined effect of SV and Li (5.0µM of SV+5.0 mM of Li) on decreasing cell viability of PC3 cells in apoptosis test (37.8 ± 19.4%) was not significantly different from the experimental value (40.8 ± 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 with 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 ± 14.9% which was not significantly different from the experimental FI (37.85 ± 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 with 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 ± 16.02%) was not significantly different from the experimental value (EFI = 41.28 ± 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, HDACIs, GSK3BIs, and mTOR inhibitors was the focus of cancer targeted therapy. Nevertheless, toxicity for the normal cells and drug resistance have limited their use.[18] 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.[19] The potency of SV[20] and Li[21] 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 HDACIs 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 metavert, 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.[22] 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.[23] 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.[24] 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.[25] 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 antiapoptotic 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.[25] 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 µM) or Li (5mM) had little or no significant effects on PC3 cells apoptosis, treatment with the combination of SV (5.0 µM) and Li (5.0 mM) increased the percentage of apoptotic cells compared with 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.[26] The apoptotic effects of SV have been attributed to the same mechanism.[27] 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.[28] 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.[29] 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.[30] 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.[31] 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 antiangiogenesis therapy has emerged as a promised method in PCa therapy.[32] Recently, Afzal et al.[33] 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

None declared.

References

1. Barsouk A, Padala SA, Vakiti A, Mohammed A, Saginala K, Thandra KC, et al. Epidemiology, staging and management of prostate cancer. *Med Sci (Basel)*. 2020;8(3):28. doi: 10.3390/medsci8030028.
2. Nguyen DD, Berlin A, Matthew AG, Perlis N, Elterman DS. Sexual function and rehabilitation after radiation therapy for prostate cancer: a review. *Int J Impot Res.* 2021;33(4):410-7. doi: 10.1038/s41443-020-00389-1.
3. Kretschmer A, Ploussard G, Heidegger I, Tsaur I, Borgmann H, Surcel C, et al. Health-related quality of life in patients with advanced prostate cancer: A systematic review. *Eur Urol Focus*. 2021;7(4):742-51. doi: 10.1016/j.euf.2020.01.017.
4. Rodrigues DN, Butler LM, Estelles DL, de Bono JS. Molecular pathology and prostate cancer therapeutics: from biology to bedside. *J Pathol*. 2014;232(2):178-84. doi: 10.1002/path.4272.
5. Sathianathen NJ, Koschel S, Thangasamy IA, Teh J, Alghazo O, Butcher G, et al. Indirect comparisons of efficacy between combination approaches in metastatic hormone-sensitive prostate cancer: a systematic review and network meta-analysis. *Eur Urol*. 2020;77(3):365-72. doi: 10.1016/j.eururo.2019.09.004.
6. Zarei MA, Dehbidi GR, Takhshid MA. Combination of NDRG2 overexpression, X-ray radiation and docetaxel enhances apoptosis and inhibits invasiveness properties of LNCaP cells. *Urol Oncol.* 2020;38(11): 849.e1-849.e9. doi: 10.1016/j.urolonc. 2020.06.017.
7. Verza FA, Das U, Fachin AL, Dimmock JR, Marins M. Roles of histone deacetylases and inhibitors in anticancer therapy. *Cancers (Basel)*. 2020;12(6):1664. doi: 10.3390/cancers12061664.
8. Ribatti D, Tamma R. Epigenetic control of tumor angiogenesis. *Microcirculation.* 2020;27(3):e12602. doi: 10.1111/micc.12602.
9. Song Y, Shiota M, Tamiya S, Kuroiwa K, Naito S, Tsuneyoshi M. The significance of strong histone deacetylase 1 expression in the progression of prostate cancer. *Histopathology.* 2011;58(5):773-80. doi: 10.1111/j.1365-2559.2011.03797.x.
10. Suenaga M, Soda H, Oka M, Yamaguchi A, Nakatomi K, Shiozawa K, et al. Histone deacetylase inhibitors suppress telomerase reverse transcriptase mRNA expression in prostate cancer cells. *Int J Cancer.* 2002;97(5):621-5. doi: 10.1002/ijc.10082.
11. Qian X, Ara G, Mills E, LaRochelle WJ, Lichenstein HS, Jeffers M. Activity of the histone deacetylase inhibitor belinostat (PXD101) in preclinical models of prostate cancer. *Int J Cancer.* 2008;122(6):1400-10. doi: 10.1002/ijc.23243.
12. Li B, Thrasher JB, Terranova P. Glycogen synthase kinase-3: a potential preventive target for prostate cancer management. *Urol Oncol.* 2015;33(11):456-63. doi: 10.1016/j.urolonc.2015.05.006.
13. Edderkaoui M, Chheda C, Soufi B, Zayou F, Hu RW, Ramanujan VK, et al. An inhibitor of GSK3B and HDACs kills pancreatic cancer cells and slows pancreatic tumor growth and metastasis in mice. *Gastroenterology.* 2018;155(6):1985-98.e5. doi: 10.1053/j.gastro.2018.08.028.
14. Adler JT, Hottinger DG, Kunnimalaiyaan M, Chen H. Inhibition of growth in medullary thyroid cancer cells with histone deacetylase inhibitors and lithium chloride. *J Surg Res*. 2010;159(2):640-4. doi: 10.1016/j.jss.2008.08.004.
15. Michaelis M, Doerr HW, Cinatl J Jr. Valproic acid as anti-cancer drug. *Curr Pharm Des.* 2007;13(33):3378-93.
16. Saberzadeh J, Omrani M, Takhshid MA. Protective effects of nimodipine and lithium against aluminum-induced cell death and oxidative stress in PC12 cells.

Iran J Basic Med Sci. 2016;19(11):1251-7.
17. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacology research & perspectives. *Pharmacol Res Perspect.* 2015;3(3):e00149. doi: 10.1002/prp2.149.
18. Baldo BA, Pham NH. Targeted drugs for cancer therapy: Small molecules and monoclonal antibodies. *Pharmacol Res Perspect.* 2015;3(3):e00149. doi: 10.1002/prp2.149.
19. Mokhtari RB, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, et al. Combination therapy in combating cancer. *Oncotarget.* 2017;8(23):38022-43. doi: 10.18632/oncotarget.16723.
20. Rana Z, Diermeier S, Hanif M, Rosengren RJ. Understanding failure and improving treatment using HDAC inhibitors for prostate cancer. *Biomedicines.* 2020;8(2):22. doi: 10.3390/biomedicines8020022.
21. Tavora F, Lotan T, Alves M, Zhou L, Amin A, Arunasalam N, et al. Glycogen synthase kinase 3-β expression in prostate cancer (PCa) correlates with aggressive pathological features and its blockade with 9-ING-41 inhibits viability of PCa cell lines. *Cancer Res.* 2020;80(16_Supplement): 2959. doi: 10.1158/1538-7445.AM2020-2959.
22. Furuta T, Sabit H, Dong Y, Miyashita K, Kinoshita M, Uchiyama N, et al. Biological basis and clinical study of glycogen synthase kinase-3β-targeted therapy by drug repositioning for glioblastoma. *Oncotarget.* 2017;8(14):22811-24. doi: 10.18632/oncotarget.15206.
23. Erguven M, Oktem G, Kara AN, Bilir A. Lithium chloride has a biphasic effect on prostate cancer stem cells and a proportional effect on midkine levels. *Oncol Lett.* 2016;12(4):2948-55. doi: 10.3892/ol.2016.4946.
24. Azimian-Zavareh V, Hossein G, Janzamin E. Effect of lithium chloride and antineoplastic drugs on survival and cell cycle of androgen-dependent prostate cancer LNCap cells. *Indian J Pharmacol*. 2012;44(6):714-21. doi: 10.4103/0253-7613.103265.
25. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res.* 2011;30(1):87. doi: 10.1186/1756-9966-30-87.
26. Kaufmann L, Marinescu G, Nazarenko I, Thiele W, Oberle C, Sleeman J, et al. LiCl induces TNF-α and FasL production, thereby stimulating apoptosis in cancer cells. *Cell Commun Signal*. 2011;9:15. doi: 10.1186/1478-811X-9-15.
27. Angelucci A, Valentini A, Millimaggi D, Gravina GL, Miano R, Dolo V, et al. Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathways. *Anticancer Drugs.* 2006;17(10):1141-50. doi: 10.1097/01.cad.0000236302.89843.fc.
28. Cunningham D, You Z. In vitro and in vivo model systems used in prostate cancer research. *J Biol Methods.* 2015;2(1):e17. doi: 10.14440/jbm.2015.63.
29. Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis. *Genes Cancer.* 2011;2(4):466-74. doi: 10.1177/1947601911408889.
30. Kiani A, Kamankesh M, Vaisi-Raygani A, Moradi MR, Tanhapour M, Rahimi Z, et al. Activities and polymorphisms of MMP-2 and MMP-9, smoking, diabetes and risk of prostate cancer. *Mol Biol Rep.* 2020;47(12):9373-83. doi: 10.1007/s11033-020-05968-5.
31. Medina-González A, Eiró-Díaz N, Fernández-Gómez JM, Ovidio-González L, Jalón-Monzón A, Casas-Nebra J, et al. Comparative analysis of the expression of metalloproteases (MMP-2, MMP-9, MMP-11 and MMP-13) and the tissue inhibitor of metalloprotease 3 (TIMP-3) between previous negative biopsies and radical prostatectomies. *Actas Urol Esp.* 2020;44(2):78-85. doi: 10.1016/j.acuro.2019.10.004. .
32. Melegh Z, Oltean S. Targeting angiogenesis in prostate cancer. *Int J Mol Sci.* 2019;20(11):2676. doi: 10.3390/ijms20112676.
33. Afzal E, Alinezhad S, Khoshnood MJ, Takhshid MA. Effects of two-by-two combination therapy with valproic acid, lithium chloride, and celecoxib on the angiogenesis of the chicken chorioallantoic membrane. *Iran J Med Sci.* 2018;43(5):506-13.