Molecular Biology Reports (2022) 49:4873–4884
https://doi.org/10.1007/s11033-022-07343-y

ORIGINAL ARTICLE

# The synergistic anticancer effect of salinomycin combined with cabazitaxel in CD44+ prostate cancer cells by downregulating wnt, NF-κB and AKT signaling

Suat Erdogan[1] · Riza Serttas[1] · Kader Turkekul[1] · Ilker Dibirdik[2]

Received: 31 August 2021 / Accepted: 8 March 2022 / Published online: 15 June 2022


## Abstract

**Background** Tumor-initiating or cancer stem cells (CSCs) reduce the effectiveness of conventional therapy. Thus, it is crucial to eliminate CSCs while killing bulky cancer cells using a combination of conventional chemotherapy and anti-CSC drugs. Salinomycin is a selective inhibitor against CSCs and shows promise in combination applications. The aim of the study was to examine the efficacy of co-administered cabazitaxel and salinomycin on the survival of prostate cancer cells and CSCs.

**Methods and Results** CD44 + stem cells were isolated from human PC3 prostate cancer cells by using magnetic activated cell sorting. The cells were concomitantly exposed to salinomycin and cabazitaxel, and the cell survival was determined by MTT test. Apoptosis was assessed by image-based cytometer, and cell migration was evaluated by wound healing assay. The expression of target mRNA and protein were assessed by RT-qPCR and Western blot, respectively. Combination index (CI) analysis showed that simultaneous administration of salinomycin and cabazitaxel was able to exert strong synergistic effect on CD44 + subpopulation (CI = 0.33), but no synergism was observed in PC3 cells. The combination of the two agents significantly increased Bax, cytochrome c, caspase-3 and − 8 mRNA expression in CD44 + CSCs, causing apoptosis. The applied therapy strategy strongly inhibited the phosphorylation of Akt, protein expression of Akt1, NF-κB and Wnt.

**Conclusions** In conclusion, our data suggest that combining salinomycin with cabazitaxel shows promise as a prostate cancer treatment approach that can target CSCs.

**Keywords** Apoptosis · Cabazitaxel · Cancer stem cell · CD44 · Prostate cancer · Salinomycin

## Introduction

Cancer is the second leading cause of death worldwide after cardiovascular disease, and chemotherapy is one of the most important trends in cancer treatment. Prostate cancer is the fifth most common malignancy in man, accounting for an estimated 375,304 deaths occurred in 2020 [1]. The prolongation of human life and the increase in the world population has increased the incidence of prostate cancer by about 40% over the last decade [2]. Androgen deprivation therapy (ADT) is the standard in metastatic hormone-sensitive prostate cancer. However, this approach is rarely curative, as recurrent metastatic castration-resistant tumors develop in 80–90% of patients. Recent clinical trials have shown that the use of taxon compounds, which are first-line therapy in ADT, in combination with agents such as abiraterone improves patient survival. However, the heterogeneous nature of the tumor cell mass is one of the factors that makes cancer treatment difficult.

Cancer stem cells (CSCs) are rare in tumor tissues, they can initiate a tumor, cause the tumor to grow, develop resistance to treatment, and trigger its recurrence [3]. These cells are characterized by their unlimited self-renewal capabilities as well as the ability to initiate new tumors phenotypically similar to the primary tumor [4]. The putative role of CSCs in the carcinogenesis and progression of prostate cancer

✉ Suat Erdogan
suaterdogan@trakya.edu.tr

[1] Department of Medical Biology, School of Medicine, Trakya University, Balkan Campus, Edirne, Turkey

[2] Department of Biochemistry, School of Medicine, Trakya University, Balkan Campus, Edirne, Turkey

may provide new insights into the biology and clinical course of prostate malignancy and enable the development of innovative therapeutic strategies [5]. Human prostate gland epithelial component is arranged from multiple stem cells that preserve the cell architecture. Prostate CSCs are differentiated from other cells by multiple properties such as various cell surface markers, self-renewal and transcription factors that generate pluripotency [6]. Relapse of the disease in some cancer patients demonstrates the importance of eliminating surviving CSCs, thus making these cells an important target in therapy.

Although numerous studies have been conducted to eliminate CSCs, effective therapeutic strategies targeting these cells have yet to be discovered. Small inhibitory molecules targeting proteins such as Wnt, Hedgehog and Notch, which play a role in CSCs growth, proliferation and self-renewal, or monoclonal antibodies targeting specific surface markers were used [7, 8]. However, these therapy strategies did not achieve significant survival or had serious side effects. Salinomycin (Fig. 1 A), an ionophore antibiotic isolated from Streptomyces albus, has been identified as a selective inhibitor of several CSCs, including prostate cancer [9]. It's mechanisms of action include reduction of ATP-binding cassette transporter expression in multidrug-resistant cells, inhibition of Akt, Wnt/β-catenin, Hedgehog and Notch signaling pathways [10, 11]. Low concentrations

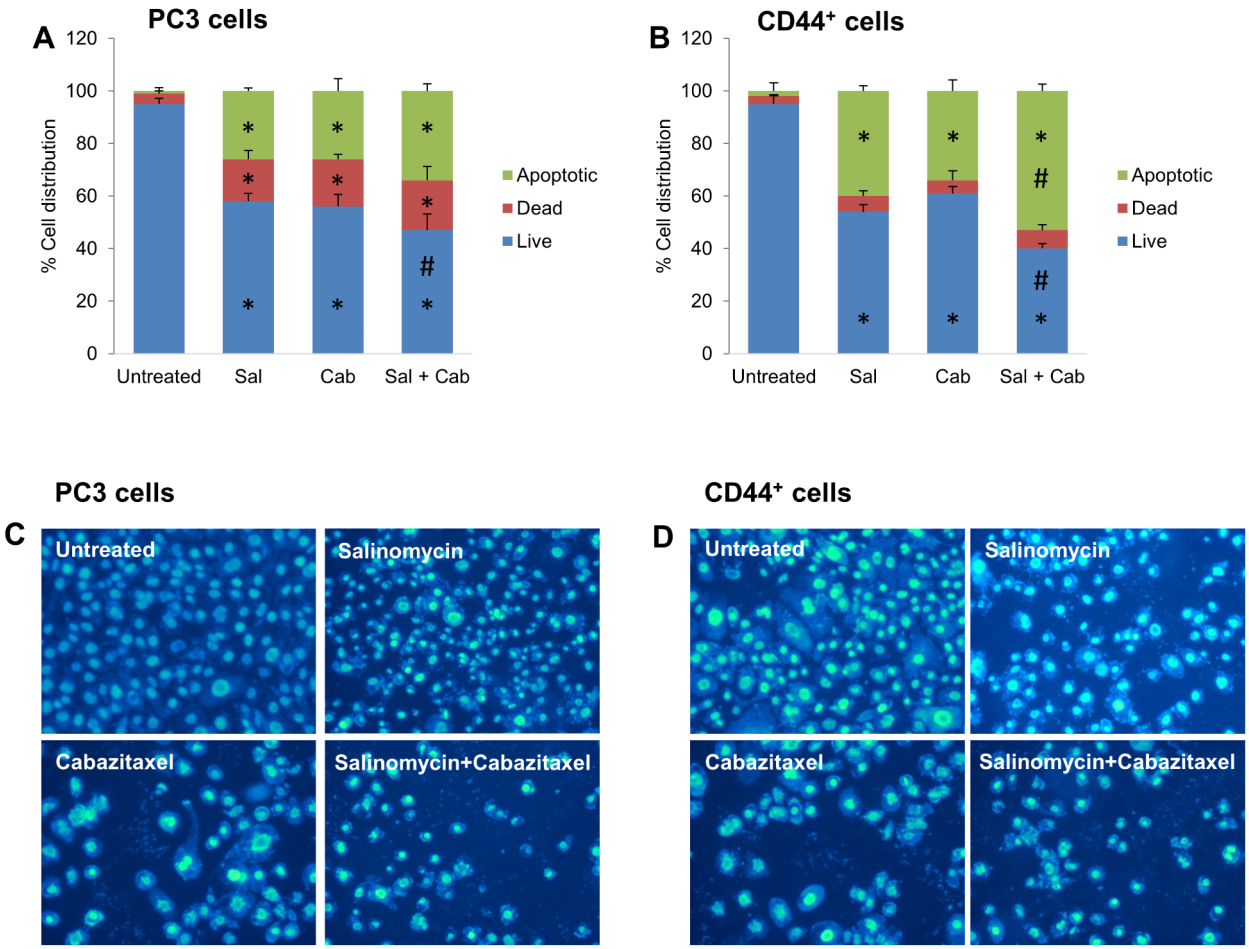


**Fig. 1** Molecular structure of salinomycin and cabazitaxel, and expression of stemness markers in the cells. The molecular structure of the natural ionophore salinomycin (A) and the taxol derivative cabazitaxel (B). The purity evaluation of isolated CD44 + cells (C). CD44, Nanong and Oct4 mRNA expressions were analyzed by RT-qPCR in isolated CD44 + CSCs and PC3s. (D). *P < 0.01 compared to PC3 cells

of salinomycin have been shown to cause apoptosis in many cancer cells by activating caspases and disrupting the imbalance of mitochondrial membrane potential [12]. As salinomycin selectively targets CSCs, its potential to eradicate drug-resistant cancer cells increases the ability of the compound to be used in therapy [10, 13].

Taxol-derived chemotherapy drugs such as paclitaxel, docetaxel and cabazitaxel are used as therapeutic agents in the treatment of metastatic prostate carcinoma [14]. Cabazitaxel (Fig. 1B) is a tubulin-binding taxane agent with antitumor activity in docetaxel-resistant cancers approved by the Food and Drug Administration for use in the treatment of hormone-refractory prostate cancer. One of the major problems with conventional chemotherapy is the risk of tumor-initiating cells becoming drug resistant and recurrent of the disease by avoiding treatment [15]. Therefore, co-administration of chemotherapeutics such as cabazitaxel with agents that are selective to tumor-initiating cancer cells may increase antitumor efficacy, allow lower use of chemotherapy agents in treatment, thereby reducing drug resistance or lessen the side effects associated with chemotherapeutics. The aim of the study was to investigate whether the combination of cabazitaxel and salinomycin has potential in the treatment of human bulky prostate cancer cells and CSCs.

## Materials and methods

### Cell Culture

Immortalized human prostate carcinoma PC3 cells were obtained from the ATCC (Manassas, VA, USA), and cultured as described previously [16]. Cells were maintained in DMEM/Ham's F-12 medium (Winsent, Quebec, Canada) supplemented with 10% fetal bovine serum (Life Technologies, USA) and penicillin/streptomycin. Cabazitaxel (Cayman, MI, USA) and salinomycin (Sigma Aldrich, MO, USA) were reconstituted in dimethyl sulfoxide (DMSO) and stored at − 20 °C until use.

### Isolation of CD44 + CSCs

CD44 + cell subpopulations were sorted by column selection using CD44-PE monoclonal antibody conjugated to magnetic microbeads (Miltenyi Biotec, Gladbach, Germany) as described previously [17]. Isolated cells were used in experiments in up to two passages in the presence of serum-free 2 ng/ml leukemia inhibitory factor, 5 ng/ml epidermal and fibroblast growth factors (Miltenyi Biotec). The purity of sorted cells was verified to be above 96% by flow cytometry (Fig. 1 C) (FACSAria, BD Biosciences, San Jose, CA, USA) [17]. In addition, RT-qPCR demonstrated that CD44 + cells express CD44, Nanog and Oct4 more potently than PC3 (Fig. 1D).

### Cell viability assay

The cytotoxic efficiency of the treatments was determined by the MTT cell viability test. For this purpose, PC3 or CD44 + cells were cultured at a density of $1 \times 10^4$ cells per 96-well plates, and incubated overnight. Subsequently, various concentrations of salinomycin (0.78 µM to 50 µM) or cabazitaxel (0.78 nM to 50 nM) were solved in fresh medium and applied to the cells for 72 h. Vehicle was added to the control cells at the concentration of the agents in the culture medium used for dissolution. After incubation, the medium was aspirated and fresh media containing 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution) (Sigma) was added, and left in the incubator for 3 h. After the MTT solution was removed, the formazan crystals formed were dissolved with DMSO. The absorbance of the wells was determined at 570 nm using a plate reader (Multiscan GO, Thermo Scientific, Vantaa, Finland). The percentage of cell viability was calculated using the following equation: optical density (OD) sample/OD blank control × 100. The efficacy of the combination was determined by simultaneous administration of 0.5 µM salinomycin and 2.5 nM cabazitaxel, doses that killed approximately half of the cells.

### Combination index analysis

The median effect analysis described by Chou and Talalay was used to determine the synergistic ratio of salinomycin and cabazitaxel [18]. The combination index (CI) score is based on the concept of dose substitution and is well suited for predicting the effects of drug combinations. CI was determined using formula CI = (D)1/(Dx)1+(D)2/(Dx)2, where (Dx)1 and (Dx)2 represents the dose of drug 1 and drug 2 in a combination which were required to achieve the same efficacy as that of drug 1 (D1) and drug 2 (D2) when used alone. PC3 cells as well as CD44 + CSCs were treated with salinomycin and cabazitaxel, either alone or in combination. Using MTT results the combination index plots was generated by CompuSyn software.

### Apoptosis detection

The types of cell deaths obtained in the treatments were quantitatively determined using an image-based cytometer. PC3 and CD44 + CSCs were seeded in 6-well plates at a density of $6 \times 10^5$ cells and incubated overnight. Cells were then to 0.5 µM salinomycin or 2.5 nM cabazitaxel

and the combination of the same concentration of the two agents for 72 h. Next, the cells were harvested and washed twice with ice-cold PBS. Alexa Fluor 488 annexin/V and propidium iodide (Invitrogen/Life Technologies, Carlsbad, CA, USA) were added to the suspended cells in annexin binding buffer (ABB), and incubated at room temperature (RT) for 15 min. Subsequently, cells were precipitated at 300 g for 5 min, resuspended in ABB and incubated for 5 min in the dark. Cell proportions were determined with an image-based cytometer (Invitrogen/Life Technologies, CA, USA) [19].

In addition to the Annexin V test, Hoechst 33,342 (Sigma) dye was also applied to distinguish apoptotic cells from healthy or necrotic cells. Briefly, CD44 + cells were cultured in a 12-well flat bottom plate overnight and exposed to 0.5 µM salinomycin or 2.5 nM cabazitaxel and combination of the two agents for 72 h. Subsequently, cells washed with PBS were dried on slides and their nuclei were stained for 10 min. Apoptotic cells characterized by apoptotic bodies, whose nucleic acids are fragmented or condensed, were photographed at 340 × 510 nm under fluorescence microscopy (ZEISS, Axio Vert A.1, Germany) (40 x) [20].

## mRNA expression analysis

The mRNA expression changes of selective apoptosis-related genes were evaluated by real-time qPCR. After incubation of CD44 + cells with 0.5 µM salinomycin, 2.5 nM cabazitaxel or their combination for 72 h, total RNA samples were isolated according to the supplier's instructions (Thermo Fisher Scientific, MA, USA). High purity RNA samples were converted to cDNA with a synthesis kit (Thermo Fisher Scientific) then amplified (Applied Biosystems, Foster City, CA) using specific primers and SYBR green (Thermo Fisher Scientific). The PCR conditions were as follows: 5 min of denaturation at 95 °C followed by 40 cycles: 15 s at 95 °C, 45 s at 60 °C, 15 s at 72 °C. The quantification of fold enrichments of targets relative to $2^{-\Delta\Delta Ct}$ was calculated using the expression GAPDH used as an internal reference. The primer pairs used in this study was shown in Table 1 (PRZ Biotech, Ankara, Turkey).

## Western blot analysis

After cells were treated with 0.5 µM salinomycin, 2.5 nM cabazitaxel or their combinations for 72 h, protein samples were prepared using lysis buffer containing protease inhibitor cocktail, sodium orthovanadate and PMSF (Thermo Fisher Scientific). For Western blot analyses, 50 µg of protein from each lysate were separated on 8–12% polyacrylamide gel, and then transferred to a PVDF membrane (Life Technologies). Membranes were incubated with NF-κB, Wnt-10a and anti-β-actin (Novus Biologicals, Littleton, CO, USA), p-Akt (Thr 308) or Akt1 (Santa Cruz Biotechnology Inc. Santa Cruz, CA) primary antibodies overnight at 4 °C. Anti-β-actin was used as an internal control. Bound antibodies were visualized using the chemiluminescence substrate kit and the appropriate immunoglobulin G (Thermo Fisher Scientific). The intensity of the protein bands was determined in the gel imaging system (Bio-Rad ChemiDoc MP System, Carlsbad, CA) [21].

**Table 1** The forward (F) and reverse (R) primer sequence used in RT-qPCR analyses.

| Target gene | Accession number | Primer sequence |
|---|---|---|
| ***CD44*** | M_017018585.1 | F: 5'-TCCCAGACGAAGACAGTCCCTGGAT-3' |
| | | R: 5'-CACTGGGGTGGAATGTGTCTTGGTC-3' |
| ***Nanog*** | NM_001297698.1 | F: 5'-ATGCCTCACACGGAGACTGT-3' |
| | | R: 5'-AGGGCTGTCCTGAATAAGCA-3' |
| ***Oct4*** | NM_001285987.1 | F: 5'-ACATGTGTAAGCTGCGGCC-3' |
| | | R: 5'-GTTGTGCATAGTCGCTGCTTG-3' |
| ***Bax*** | XM_017027077.1 | F: 5′-TTGCTTCAGGGTTTCATCCA-3' |
| | | R: 5′-CAGCCTTGAGCACCAGTTTG-3' |
| ***Caspase 3*** | NM_001354784.1 | F: 5′-CAAACTTTTTCAGAGGGGATCG-3' |
| | | R: 5'-GCATACTGTTTCAGCATGGCA-3' |
| ***Caspase 8*** | NM_001080125.1 | F: 5'-CTGCTGGGGATGGCCACTGTG-3' |
| | | R: 5'-TCGCCTCGAGGACATCGCTCTC-3' |
| ***Cytochrome c*** | NM_018947.5 | F:5'-AGTGGCTAGAGTGGTCATTCATTTACA-3' |
| | | R: 5'-TCATGATCTGAATTCTGGTGTATGAGA-3' |
| ***p53*** | NM_001126118.1 | F: 5'-GAGGTTGGCTCTGACTGTACC-3' |
| | | R: 5'-TCCGTCCCAGTAGATTACCAC-3' |
| ***GAPDH*** | NM_001289745.2 | F: 5'-TTGGTATCGTGGAAGGACTCA-3' |
| | | R: 5'-TGTCATCATATTTGGCAGGTTT-3' |

## Statistical analysis

Statistical data were evaluated with SPSS software (19.0; SPSS, Chicago, IL), the differences between treatment groups were determined by analysis of variance (ANOVA), followed by Duncan's multiple range test. Results were expressed as mean ± standard deviation (SD), and each experiment was performed at least three times. $P < 0.05$ was considered statistically significant.

# Results

## Salinomycin combined with cabazitaxel synergistically inhibits CD44 + cancer stem cells

To examine the effect of the pharmacological agents used on PC3 and CD44 + CSCs, cells were treated with 0.78–50 µM salinomycin (Fig. 2 A, B) and 0.78–50 nM cabazitaxel (Fig. 2 C, D) for 72 h. Both agents dose-dependently

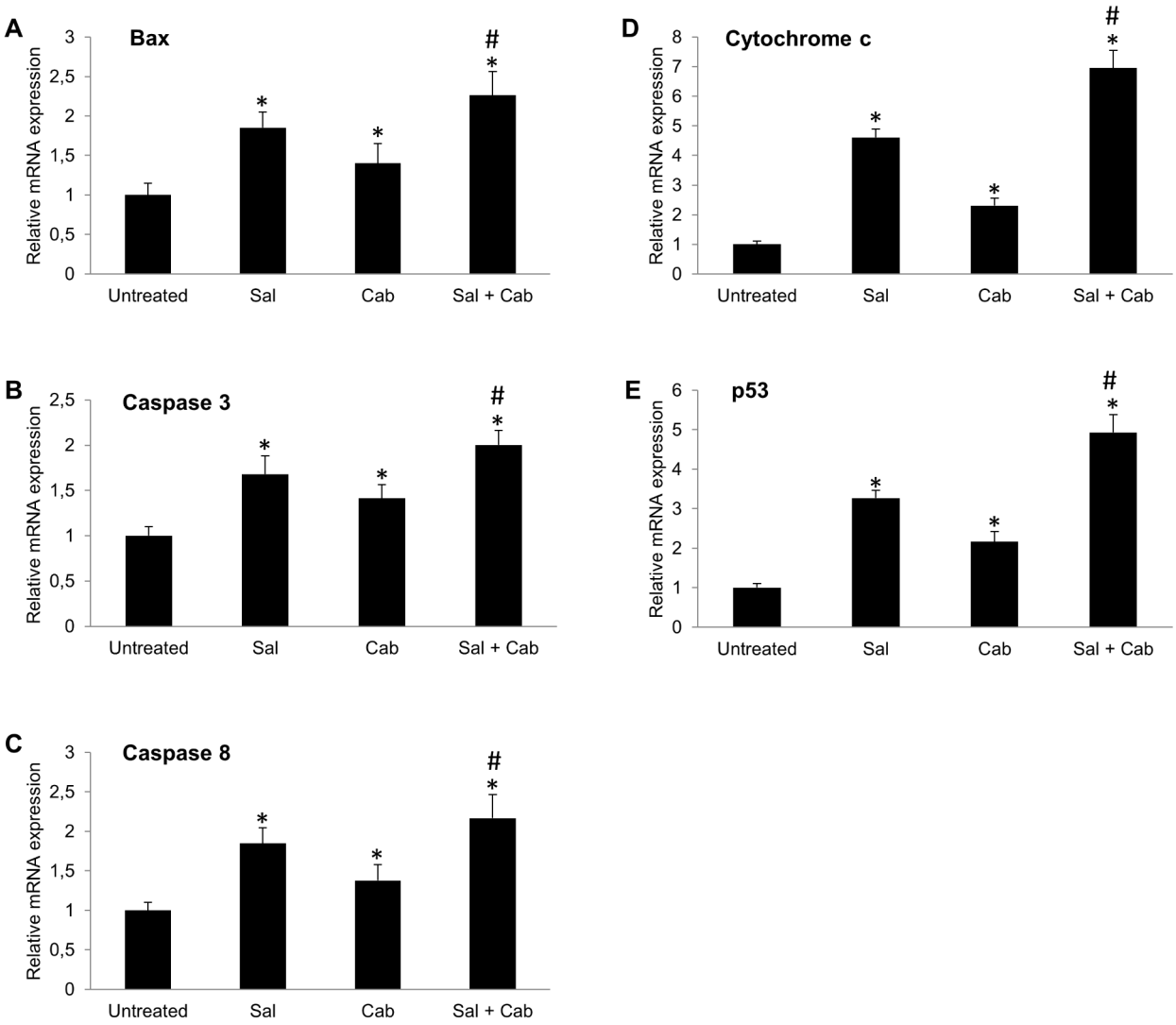


**Fig. 2** The combination of salinomycin and cabazitaxel significantly inhibits PC3 and CD44 + cell survival. Administration of salinomycin (A, B) and cabazitaxel (C, D) dose-dependently inhibit survival in both PC3 and CD44 + cells. Cells were exposed to various concentrations of agents for 72 h. PC3 (E) and CD44 + cells (F) were treated with 0.5 µM salinomycin (Sal), 2.5 nM cabazitaxel (Cab) or the same combination of the two agents (Sal + Cab) for 72 h. Subsequently, cell viability was determined by MTT assay and cell survivals were expressed as a percentage relative to control cells. *$P < 0.01$ compared to untreated control cells. **$P < 0.05$ compared to Sal or Cab. #$P < 0.01$ compared to Sal or Cab

inhibited PC3 and CD44 + CSCs survival. Concomitant administration of salinomycin and cabazitaxel caused significantly more death in both cell types than single treatment (Fig. 2E, F). However, drug interaction values in CD44 + cells were found less than 1 (CI = 0.33) whereas the CI values in PC3 was more than 1 (CI = 1.03). Thus, the combination of salinomycin and cabazitaxel showed strong synergism in CD44 + cells ($p < 0.01$), while the effect in PC3 cells was nearly additive ($p < 0.05$) [28].

## The combination leads to higher apoptosis than single administration

The impact of treatments on apoptotic cell death was determined by Annexin V/PI analysis. Accordingly, the addition of salinomycin to cabazitaxel treatment significantly increased the percentage of apoptotic cells compared to a single application. Both salinomycin and cabazitaxel cause apoptosis in PC3 cells at a rate of 26%, while combined administration increases it to 34% (Fig. 3 A). On the other hand, salinomycin and cabazitaxel induce apoptosis in CD44 + cells by 40% and 34%, respectively (Fig. 3B). In addition, administration of the two agents in combination to CD44 + cells induces apoptosis at a level of 53%, which is significantly higher than single treatments ($p < 0.01$). It is noteworthy that dual application or single treatments were more effective in CD44 + cell death than PC3 cells ($p < 0.05$). Non-apoptotic cell death was higher in the treatment groups than in untreated PC3 cells, but there was no difference between the treatment regimens (Fig. 3 A). There

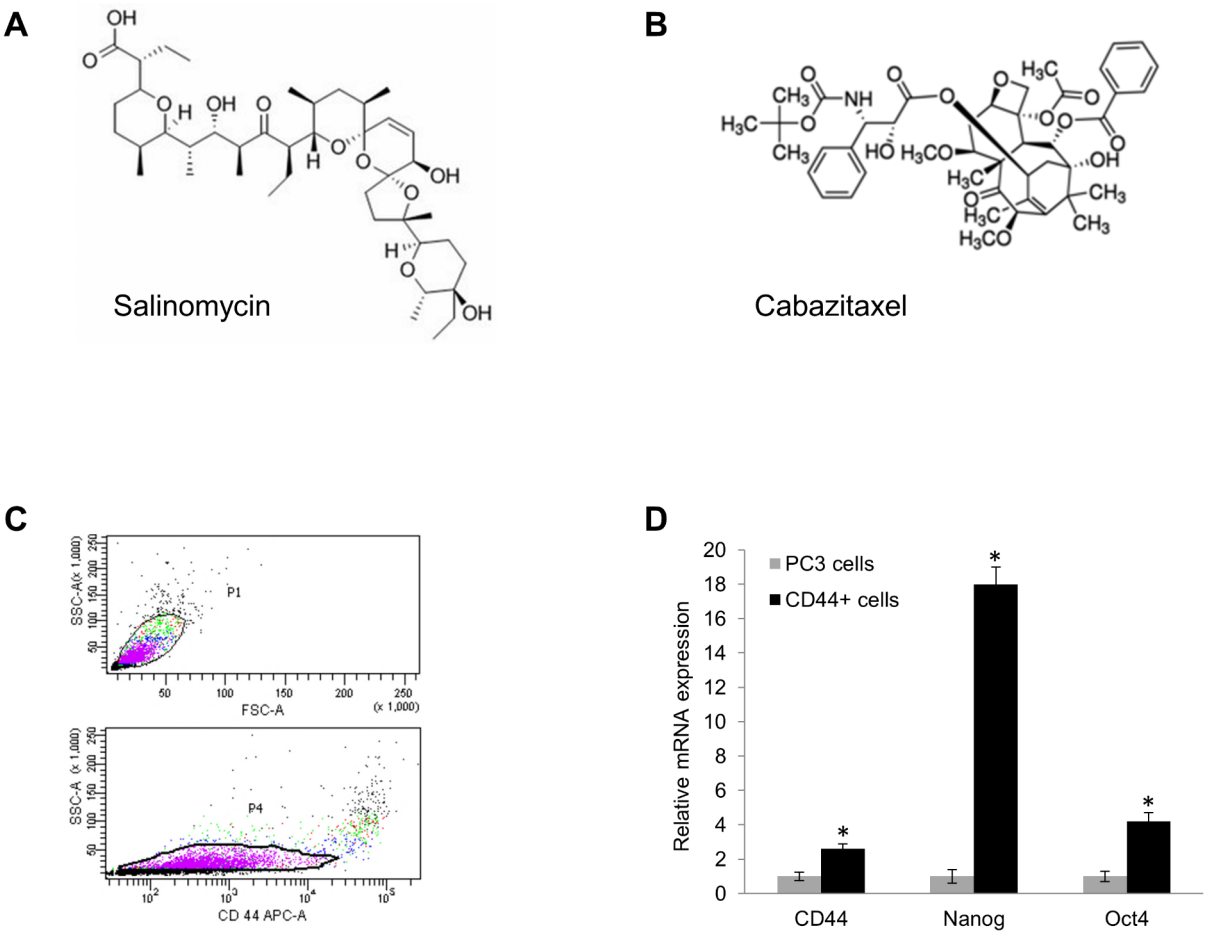


**Fig. 3** Co-administration of salinomycin and cabazitaxel induces apoptosis. PC3 and CD44 + cells were incubated with 0.5 µM salinomycin (Sal), 2.5 nM cabazitaxel (Cab) or combination of the agents (Sal + Cab) for 72 h. Then, cell-based cytometric assay was performed using AnnexinV/PI (A, B). After similar treatment, cells were stained with Hoechst 33,342 and apoptotic cells were observed under fluorescent light (C, D). *P < 0.01 compared to untreated control. #P < 0.01 compared to Sal or Cab

was also no difference in non-apoptotic CD44 + cell death in the treatment groups compared to the untreated control (Fig. 3B). Although not quantitatively, we also demonstrated the presence of apoptosis in CD44 + cells by Hoechst staining, accordingly apoptotic bodies were much more prominent in the treatment groups (Fig. 3 C, 3D).

### Co-treatment induces mRNA expression of apoptosis-associated genes

To evaluate the molecular mechanisms of combination therapy in CD44 + CSCs, mRNA expression of selected apoptosis-related genes was determined. Single application of both salinomycin and cabazitaxel significantly increased the mRNA expression of Bax, caspase 3, caspase 8, cytochrome c and p53 (Fig. 4 A – 4E). Dual treatment strongly upregulated mRNA expression of all selected genes compared to single treatments.

### Combination therapy alters protein expression

The effect of the treatment modalities on the expression of proteins involved in proliferation, differentiation, self-renewal of cancer and cancer stem cells were evaluated by Western blot. Salinomycin administration significantly down regulated the p105 subunits of NF-κB in both cells compared to untreated control (Fig. 5 A, 5B), while cabazitaxel strongly suppressed in CD44 + cells but ineffective in PC3 cells (Fig. 5 A, B). Salinomycin significantly inhibited the expression of the p50 subunit in PC3 cells (Fig. 5 C) but did not alter it in CD44 + cells (Fig. 5D). Conversely, cabazitaxel was ineffective on p50 in PC3 cells (Fig. 5 C), but significantly suppressed it in CD44s (Fig. 5D). Remarkably, co-administration of the two pharmacological agents to both cells resulted in significant down-regulation of both subunits of NF-κB compared to single administrations (Fig. 5 A – 5D).

Neither salinomycin, cabazitaxel nor the combination therapy changed the expression levels of p-Akt (Fig. 5E) or Akt1 (Fig. 5G) in PC3 cells. On the other hand, both agents downregulated the phosphorylation of Akt expression in CD44 + cells (Fig. 5 F), while the combined treatment downregulated Akt phosphorylation at a significantly higher level than single administration (Fig. 5 F). Combined treatment of CD44 + cells suppressed Akt phosphorylation by 75% compared to Akt expression. In contrast to its effect on pAkt, cabazitaxel did not significantly alter the expression of Akt1 either in single or combined administration to CD44 + cells (Fig. 5 H). Single treatments did inhibit the protein expression of Wnt in both cells compared to untreated group (Fig. 5I J). Likewise, the combination treatment downregulated the expression of Wnt more potently than the single administration in both cell types.

## Discussion

Cabazitaxel is a semi-synthetic derivative of docetaxel and is a chemotherapeutic agent used in the second-line treatment of metastatic castration-resistant prostate cancer [14]. However, the usefulness of the agent is limited due to side effects such as diarrhea, nausea and vomiting. According to phase trial results, cabazitaxel therapy combined with carboplatin [22] and abiraterone [23] showed better clinical efficacy in men with metastatic castration-resistant prostate cancer. Completed phase studies show that a superior treatment option has not yet been developed for the treatment of prostate cancer. Therefore, the combination of a currently proven chemotherapeutic drug with another agent with a different type of action may lead to the development of a new treatment strategy.

Targeting CSCs may have an important strategy in treatment. Such cells, which can initiate cancer, may develop resistance to treatment and cause the disease to recur [3, 24]. The success rate in therapy applications that target CSCs as well as bulky tumors is more effective than other treatment strategies [25, 26]. The combination of salinomycin and docetaxel has recently been reported to be effective in the treatment of breast [26], cervix [25] and gastric [27] cancers. To date, the combination of salinomycin has not been tested for the potential of neither docetaxel nor cabazitaxel in prostate cancer or their CSCs. Thus, here cabazitaxel and salinomycin were combined and used in PC3 and CD44 + CSC therapies. Our findings showed that co-administration of the two agents reduces PC3 and CSC survival by 16% and 33% more than single administration of the chemotherapeutic agent, respectively. Moreover, the combination therapy produces a synergistic effect on CD44 + cells, whereas this effect does not occur on PC3 cells. According to the results of this study, salinomycin co-administered with cabazitaxel significantly increases the efficacy of treatment, consistent with previous taxol derivative docetaxel results [26, 25]. The results showed that the combination therapy had a stronger cytotoxic effect on CD44 + than on PC3 cells; may suggest that the treatment strategy demonstrates cancer stem cell selectivity.

The mechanisms in the selectivity of salinomycin against CSCs are not fully understood. However, among the known effects, salinomycin increases intracellular reactive oxygen radical (ROS) levels, and inhibits cancer cell survival by causing endoplasmic reticulum stress [29]. These events are followed by reduction in mitochondrial membrane potential, Bax translocation from mitochondria, release of cytochrome c into cytoplasm and activation of the caspase-3 [30, 9]. Finally, cells whose intracellular balance is disturbed by salinomycin mostly die due to apoptosis [29]. In this study, it was shown that a single administration of salinomycin

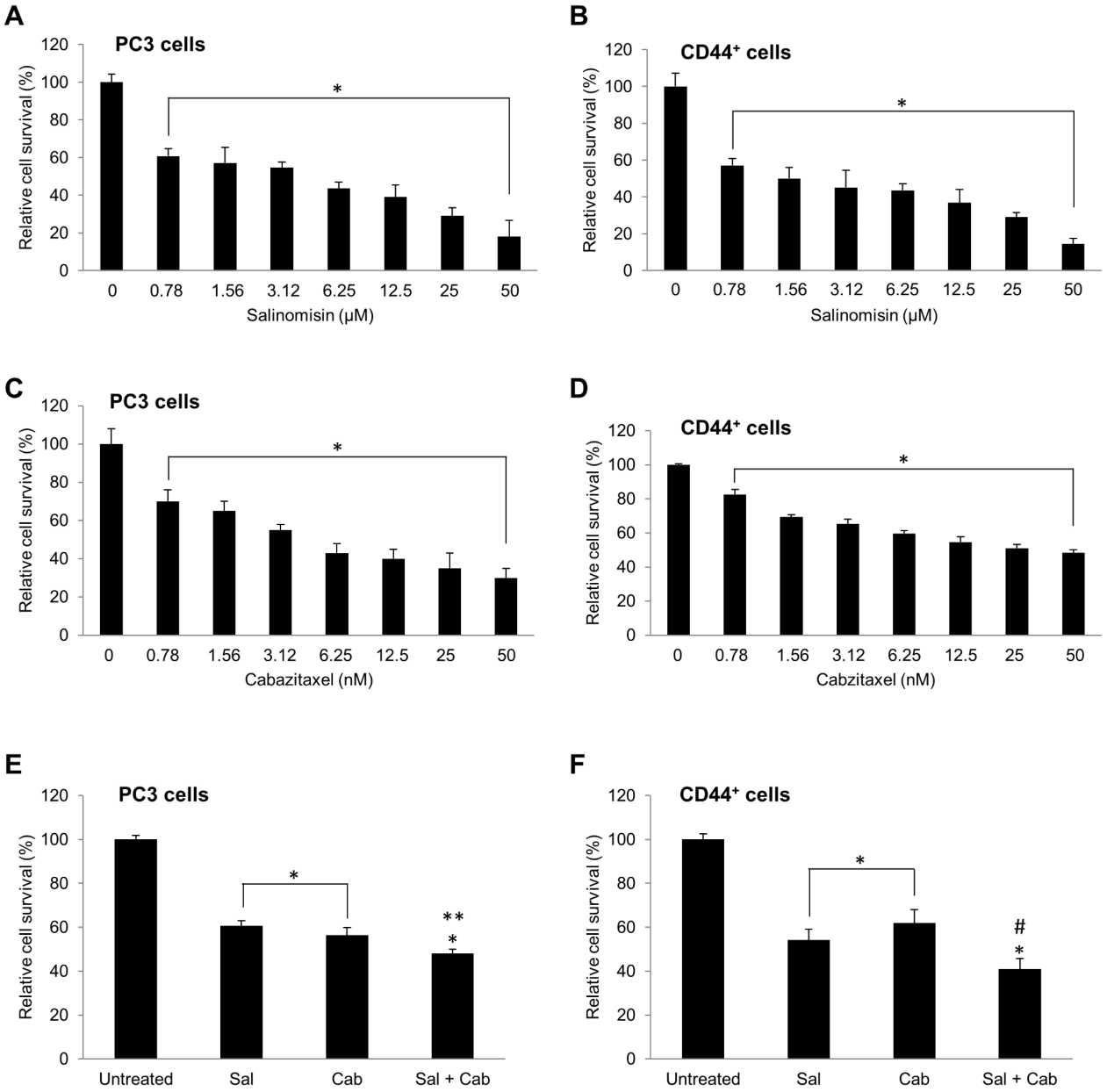


**Fig. 4** Dual treatment induces mRNA expression of apoptosis-related genes. Isolated CD44 + cells were treated with 0.5 μM salinomycin (Sal), 2.5 nM cabazitaxel (Cab) or combination of the agents (Sal + Cab) for 72 h. Then, cDNAs were synthesized from the extracted mRNA samples and qPCR was performed using Bax (A), caspase 3 (B), caspase 8 (C), cytochrome c (D) and p53 (E) specific primers. *$P < 0.01$ compared to untreated control. #$P < 0.01$ compared to Sal or Cab

significantly induced apoptosis in both PC3 and CSCs. The fact that the percentage of apoptotic cells formed was higher in CSCs than PC3 cells supports the selectivity of salinomycin to CSCs. It has been proven in previous studies that salinomycin or derivatives shows selectivity to CSCs [31, 13] and low toxicity to non-malignant prostate cells [9]. Addition of salinomycin to cabazitaxel treatment did not significantly alter the apoptotic rate induced by single administration in PC3 cells, but strongly increased it in CSCs. Although the combination did not change the percentage of apoptosis in parent PC3 cells compared to a single application, it decreased cell survival by inducing

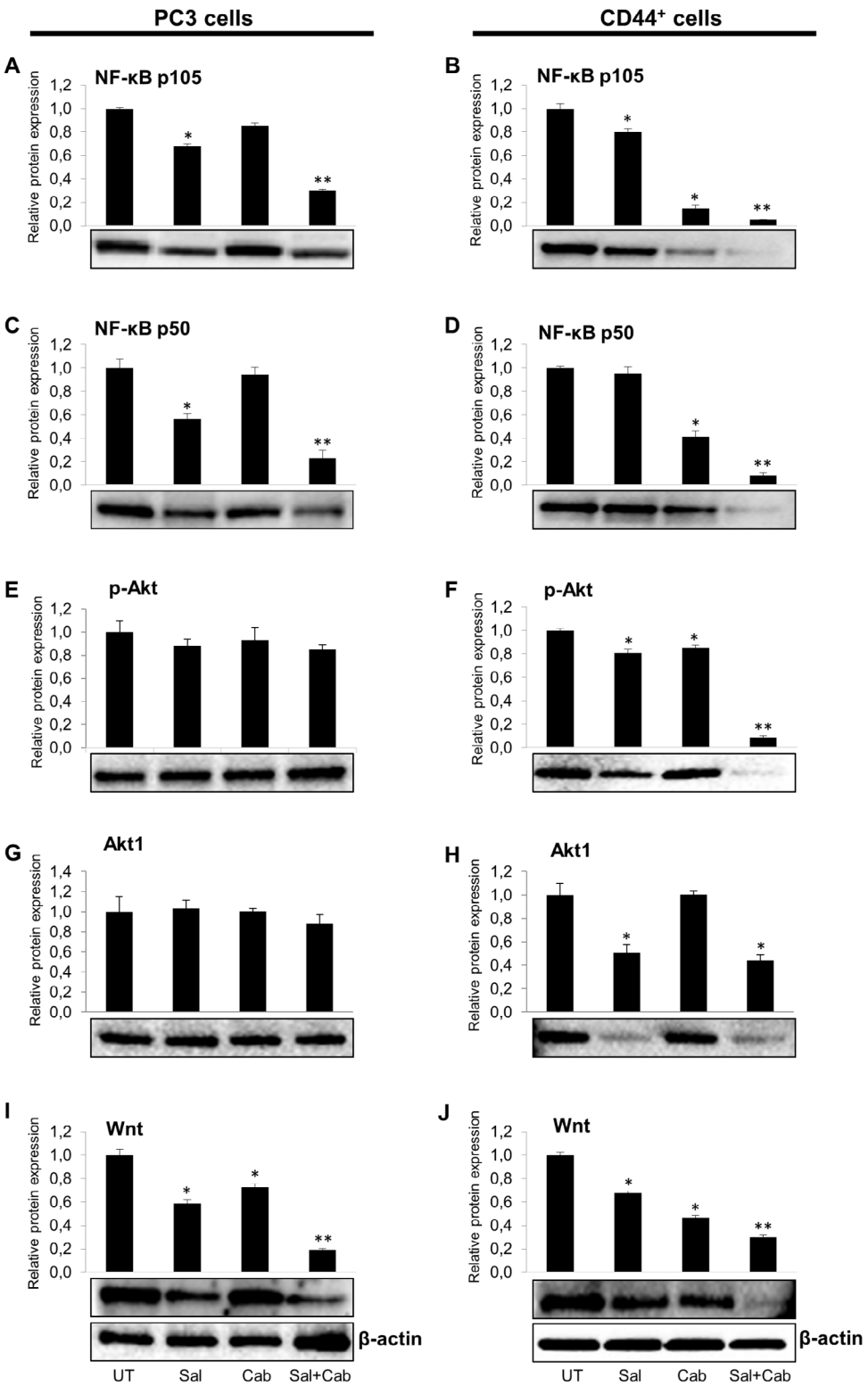


**Fig. 5** Treatment strategies alter signaling pathways involved in cell proliferation. PC3 or CD44 + cells were incubated with 0.5 µM salinomycin (Sal), 2.5 nM cabazitaxel (Cab), combination of the agents (Sal + Cab) or left untreated (UT) for 72 h. After the protein samples were extracted, their expression was analyzed by Western blot using antibodies specific for NF-κB p105 (A – B), NF-κB p50 (C - D), p-Akt (E - F), Akt1 (G – H) and Wnt (I - J) proteins. β-actin protein expression was determined to compare relative protein levels between samples. *P < 0.01 compared to untreated control. **P < 0.01 compared to Sal or Cab

non-apoptotic cell death compared to the control group. This result indicates that salinomycin may have induced one of the non-apoptotic cell deaths, such as necrosis or autophagy. The report that salinomycin causes cell death in cultured glioma cells, mainly by inducing programmed necrosis through ROS production supports our data [32]. The increased expression of Bax, caspase 3, caspase 8 and cytochrome c in CSCs of combined treatment compared to single applications may explain the mechanisms of apoptosis occurring in dual application [12]. The fact that salinomycin treatment induces CSCs to higher levels of apoptosis may be explained by the higher expression of p-Akt by these cells, which salinomycin significantly inhibits. According to the current results and previous reports, CSCs express higher levels of Akt1/2 than their parent cells [33]. Therefore, administration of salinomycin alone or in combination with cabazitaxel may have increased the apoptosis of CSCs as a result of the downregulation of Akt expression.

It has been well established that salinomycin has selective activity against CSCs by suppressing Wnt/β-catenin, Hedgehog, Notch [10, 11], Akt and NF-κB [34] signaling pathways, reducing ABC-binding transporters [11] as well as inhibiting stemness properties [35]. PC3 and CD44 + CSCs responded differently to salinomycin or cabazitaxel single treatments, while dual treatment significantly suppressed the protein expression of NF-κB in CSCs at a higher level than in PC3 cells. The efficacy of the treatment modality in CSCs may be explained by the fact that prostate CSCs express higher constitutive NF-κB activity than parental tumors [36]. On the other hand, the reason why cabazitaxel downregulates the expression of NF-κB subunits more strongly than salinomycin alone is a finding that needs clarification. Similar to the present study, it was previously shown that salinomycin inhibits the proliferation of cisplatin-resistant ovarian cancer [34], breast cancer [37] and prostate cancer cells [38] related to the inhibition of Akt/NF-κB pathways. *In vivo* studies with PC3 cells also demonstrated that salinomycin exerts its anti-tumor activity by suppressing the Wnt/β-catenin pathway and inducing apoptosis [9]. Another effect of salinomycin is to sensitize cells to antimitotic drugs by preventing G2 cell cycle arrest and increasing apoptosis, thus causing the death of cancer cells [39]. These results suggest that the combination of salinomycin with various anti-tumor drugs, such as cabazitaxel, may increase the efficacy of the treatment.

## Conclusions

In summary, concomitant administration of cabazitaxel and salinomycin provided a synergistic advantage over single administration in the treatment of castration-resistant prostate cancer. Our results therefore provided data that the addition of salinomycin to conventional chemotherapy could increase the treatment efficacy by being effective in selectively eliminating cancer-initiating stem cells.

**Acknowledgements** This study was funded by Scientific Research Projects Coordination Unit of Trakya University (Project number: 2018 – 312).

**Authors' contributions** SE and ID designed the study, SE coordinated the research and wrote manuscript. RS and KT performed the experiments. All authors read and approved the manuscript.

**Funding** This study was funded by Scientific Research Projects Coordination Unit of Trakya University (Project number: 2018 – 312).

**Availability of data and material** (data transparency): Yes.

**Code Availability** (software application or custom code): Not applicable.

**Declarations** Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals: Not applicable.

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethics approval** (include appropriate approvals or waivers): Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

## References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71(3):209–249. doi:https://doi.org/10.3322/caac.21660
2. https://doi.org/10.1001/jamaoncol.2018.2706
3. Barbato L, Bocchetti M, Di Biase A, Regad T (2019) Cancer Stem Cells and Targeting Strategies. Cells 8(8): 926. doi:https://doi.org/10.3390/cells8080926
4. Yoshida GJ, Saya H (2016) Therapeutic strategies targeting cancer stem cells. Cancer Sci 107(1):5–11. doi:https://doi.org/10.1111/cas.12817
5. Alison MR, Lim SM, Nicholson LJ (2011) Cancer stem cells: problems for therapy? J Pathol 223(2):147–161. doi:https://doi.org/10.1002/path.2793
6. Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis J, Kretsovali A (2015) Common stemness regulators of embryonic and cancer stem cells. World J Stem Cells 7(9):1150–1184. doi:https://doi.org/10.4252/wjsc.v7.i9.1150
7. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, Zhang G, Wang X, Dong Z, Chen F, Cui H (2020) Targeting cancer stem cell pathways for cancer therapy. Signal Transduct Target Ther 5(1):8. doi:https://doi.org/10.1038/s41392-020-0110-5

8. Fendler A, Bauer D, Busch J, Jung K, Wulf-Goldenberg A, Kunz S, Song K, Myszczyszyn A, Elezkurtaj S, Erguen B, Jung S, Chen W, Birchmeier W (2020) Inhibiting WNT and NOTCH in renal cancer stem cells and the implications for human patients. Nat Commun 11(1):929. doi:https://doi.org/10.1038/s41467-020-14700-7
9. Zhang Y, Liu L, Li F, Wu T, Jiang H, Jiang X, Du X, Wang Y (2017) Salinomycin Exerts Anticancer Effects on PC-3 Cells and PC-3-Derived Cancer Stem Cells In Vitro and In Vivo. Biomed Res Int 2017:4101653. doi:https://doi.org/10.1155/2017/4101653
10. Liu Q, Sun J, Luo Q, Ju Y, Song G (2021) Salinomycin Suppresses Tumorigenicity of Liver Cancer Stem Cells and Wnt/Beta-catenin Signaling. Curr Stem Cell Res Ther 16(5):630–637. doi:https://doi.org/10.2174/1574888X15666200123121225
11. Naujokat C, Steinhart R (2012) Salinomycin as a Drug for Targeting Human Cancer Stem Cells. J Biomed Biotechnol. https://doi.org/10.1155/2012/950658
12. Kim KY, Yu SN, Lee SY, Chun SS, Choi YL, Park YM, Song CS, Chatterjee B, Ahn SC (2011) Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. Biochem Bioph Res Co 413(1):80–86. doi:https://doi.org/10.1016/j.bbrc.2011.08.054
13. Versini A, Colombeau L, Hienzsch A, Gaillet C, Retailleau P, Debieu S, Muller S, Caneque T, Rodriguez R (2020) Salinomycin Derivatives Kill Breast Cancer Stem Cells by Lysosomal Iron Targeting. Chemistry 26(33):7416–7424. doi:https://doi.org/10.1002/chem.202000335
14. Hongo H, Kosaka T, Oya M (2018) Analysis of cabazitaxel-resistant mechanism in human castration-resistant prostate cancer. Cancer Sci 109:2937–2945. doi:https://doi.org/10.1111/cas.13729
15. Kapoor A, Wu C, Shayegan B, Rybak AP (2016) Contemporary agents in the management of metastatic castration-resistant prostate cancer. Can Urol Assoc J 10(11–12):E414–E423. doi:https://doi.org/10.5489/cuaj.4112
16. Erdogan S, Doganlar O, Doganlar ZB, Turkekul K (2018) Naringin sensitizes human prostate cancer cells to paclitaxel therapy. Prostate Int 6(4):126–135. doi:https://doi.org/10.1016/j.prnil.2017.11.001
17. Erdogan S, Turkekul K (2020) Neferine inhibits proliferation and migration of human prostate cancer stem cells through p38 MAPK/JNK activation. J Food Biochem. doi:ARTNe13253 10.1111/jfbc.13253
18. Chou TC (2010) Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Res 70(2):440–446. doi:https://doi.org/10.1158/0008-5472.Can-09-1947
19. Erdogan S, Doganlar O, Doganlar ZB, Serttas R, Turkekul K, Dibirdik I, Bilir A (2016) The flavonoid apigenin reduces prostate cancer CD44(+) stem cell survival and migration through PI3K/Akt/NF-kappaB signaling. Life Sci 162:77–86. doi:https://doi.org/10.1016/j.lfs.2016.08.019
20. Erdogan S, Turkekul K, Dibirdik I, Doganlar O, Doganlar ZB, Bilir A, Oktem G (2018) Midkine downregulation increases the efficacy of quercetin on prostate cancer stem cell survival and migration through PI3K/AKT and MAPK/ERK pathway. Biomed Pharmacother 107:793–805. doi:https://doi.org/10.1016/j.biopha.2018.08.061
21. Erdogan S, Turkekul K, Serttas R, Erdogan Z (2017) The natural flavonoid apigenin sensitizes human CD44(+) prostate cancer stem cells to cisplatin therapy. Biomed Pharmacother 88:210–217. doi:https://doi.org/10.1016/j.biopha.2017.01.056
22. Corn PG, Heath EI, Zurita A, Ramesh N, Xiao LC, Sei E, Li-Ning-Tapia E, Tu SM, Subudhi SK, Wang J, Wang XM, Efstathiou E, Thompson TC, Troncoso P, Navin N, Logothetis CJ, Aparicio AM (2019) Cabazitaxel plus carboplatin for the treatment of men with metastatic castration-resistant prostate cancers: a randomised, open-label, phase 1–2 trial. Lancet Oncol 20(10):1432–1443. doi:https://doi.org/10.1016/S1470-2045(19)30408-5
23. Massard C, Mateo J, Loriot Y, Pezaro C, Albiges L, Mehra N, Varga A, Bianchini D, Ryan CJ, Petrylak DP, Attard G, Shen L, Fizazi K, de Bono J (2017) Phase I/II trial of cabazitaxel plus abiraterone in patients with metastatic castration-resistant prostate cancer (mCRPC) progressing after docetaxel and abiraterone. Ann Oncol 28(1):90–95. doi:https://doi.org/10.1093/annonc/mdw441
24. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100(7):3983–3988. doi:https://doi.org/10.1073/pnas.0530291100
25. Wang Q, Yen YT, Xie C, Liu FC, Liu Q, Wei J, Yu LX, Wang LF, Meng FY, Li RT, Liu BR (2021) Combined delivery of salinomycin and docetaxel by dual-targeting gelatinase nanoparticles effectively inhibits cervical cancer cells and cancer stem cells. Drug Deliv 28(1):510–519. doi:https://doi.org/10.1080/10717544.2021.1886378
26. Gao J, Liu JJ, Xie FY, Lu Y, Yin C, Shen X (2019) Co-Delivery of Docetaxel and Salinomycin to Target Both Breast Cancer Cells and Stem Cells by PLGA/TPGS Nanoparticles. Int J Nanomed 14:9199–9216. doi:https://doi.org/10.2147/Ijn.S230376
27. Li L, Cui DJ, Ye LM, Li Y, Zhu LY, Yang LQ, Bai BJ, Nie Z, Gao J, Cao Y (2017) Codelivery of salinomycin and docetaxel using poly(D,L-lactic-co-glycolic acid)- poly(ethylene glycol) nanoparticles to target both gastric cancer cells and cancer stem cells. Anti-Cancer Drug 28(9):989–1001. doi:https://doi.org/10.1097/Cad.0000000000000541
28. Chou TC (2008) Preclinical versus clinical drug combination studies. Leuk Lymphoma 49(11):2059–2080. doi:https://doi.org/10.1080/10428190802353591
29. Yu J, Yang Y, Li S, Meng P (2021) Salinomycin triggers prostate cancer cell apoptosis by inducing oxidative and endoplasmic reticulum stress via suppressing Nrf2 signaling. Exp Ther Med 22(3):946. doi:https://doi.org/10.3892/etm.2021.10378
30. An H, Kim JY, Lee N, Cho Y, Oh E, Seo JH (2015) Salinomycin possesses anti-tumor activity and inhibits breast cancer stem-like cells via an apoptosis-independent pathway. Biochem Biophys Res Commun 466(4):696–703. doi:https://doi.org/10.1016/j.bbrc.2015.09.108
31. Gruber M, Handle F, Culig Z (2020) The stem cell inhibitor salinomycin decreases colony formation potential and tumor-initiating population in docetaxel-sensitive and docetaxel-resistant prostate cancer cells. Prostate 80(3):267–273. doi:https://doi.org/10.1002/pros.23940
32. Qin LS, Jia PF, Zhang ZQ, Zhang SM (2015) ROS-p53-cyclophilin-D signaling mediates salinomycin-induced glioma cell necrosis. J Exp Clin Cancer Res 34:57. doi:https://doi.org/10.1186/s13046-015-0174-1
33. Yoon C, Lu J, Yi BC, Chang KK, Simon MC, Ryeom S, Yoon SS (2021) PI3K/Akt pathway and Nanog maintain cancer stem cells in sarcomas. Oncogenesis 10(1):12. doi:https://doi.org/10.1038/s41389-020-00300-z
34. Parajuli B, Lee HG, Kwon SH, Cha SD, Shin SJ, Lee GH, Bae I, Cho CH (2013) Salinomycin inhibits Akt/NF-kappaB and induces apoptosis in cisplatin resistant ovarian cancer cells. Cancer Epidemiol 37(4):512–517. doi:https://doi.org/10.1016/j.canep.2013.02.008
35. Lee HG, Shin SJ, Chung HW, Kwon SH, Cha SD, Lee JE, Cho CH (2017) Salinomycin reduces stemness and induces apoptosis on human ovarian cancer stem cell. J Gynecol Oncol 28 (2). doi:ARTN e14 10.3802/jgo.2017.28.e14

36. Rajasekhar VK, Studer L, Gerald W, Socci ND, Scher HI (2011) Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappa B signalling.Nat Commun 2. doi:ARTN 162 10.1038/ncomms1159
37. Tyagi M, Patro BS (2019) Salinomycin reduces growth, proliferation and metastasis of cisplatin resistant breast cancer cells via NF-kB deregulation. Toxicol in Vitro 60:125–133. doi:https://doi.org/10.1016/j.tiv.2019.05.004
38. Ketola K, Hilvo M, Hyotylainen T, Vuoristo A, Ruskeepaa AL, Oresic M, Kallioniemi O, Iljin K (2012) Salinomycin inhibits prostate cancer growth and migration via induction of oxidative stress. Brit J Cancer 106(1):99–106. doi:https://doi.org/10.1038/bjc.2011.530
39. Kim JH, Yoo HI, Kang HS, Ro J, Yoon S (2012) Salinomycin sensitizes antimitotic drugs-treated cancer cells by increasing apoptosis via the prevention of G2 arrest. Biochem Bioph Res Co 418(1):98–103. doi:https://doi.org/10.1016/j.bbrc.2011.12.141