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ORIGINAL ARTICLE

Vitamin D increases the efficacy of cisplatin on bladder cancer cell lines

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Abstract

Background 1,25(OH)2D3(Calcitriol), which is a broad regulatory molecule, plays a role in changing the efficacy of chemotherapeutic drugs. Cisplatin is one of a current standard chemotherapy regimen for bladder cancer. Increasing the effectiveness of the treatment and reducing the side effects to chemotherapeutics are of great importance in bladder cancer. We aimed to investigate the effect of the combination of cisplatin and calcitriol in order to create a possible advantage in treatment of bladder cancer.

Methods T24, ECV-304 and HUVEC cell lines were treated with calcitriol and cisplatin individually and in combination. Dose determination and combination treatments of calcitriol and cisplatin were evaluated using the MTT assay for cytotoxicity analysis on the cells. Annexin V-PI staining method was used for apoptosis determination by flow cytometry. Also the P-gp expression levels were determined by flow cytometry.

Results The combination treatment increased the anti-proliferative efficacy compared to the efficacy in cisplatin alone in T24 cells and reduced the cytotoxicity in the HUVEC healthy cells compared to cisplatin alone. Combination treatment achieved significantly higher apoptosis rate in T24 cells compared with the rates in treatment of cisplatin alone. However apoptosis decreased in HUVEC cell line. P-gp ratios were increased in HUVEC and decreased in T24 cells with combination treatment compared to the numbers in the control cells. The rate of apoptosis and P-gp levels showed no significant change in ECV-304 cells.

Conclusion Our study revealed that the combination of calcitriol and cisplatin allows the use of cisplatin at lower doses in T24 bladder cancer cell line.

Keywords Bladder cancer · T24 cell line · Calcitriol · Cisplatin · Vitamin D · P-gp · Apoptosis

Introduction

Variations in the rates of multiple cancers, in survival rates and variability in cancer development make it difficult to determine the incidence rates in bladder cancer. Bladder cancer is the 10th type of cancer worldwide. The incidence is four times higher in men than in women. The environmental, demographic and genetic factors are known to be effective in the development of bladder cancer. Although it is difficult to determine the rates of their effect on disease development, the incidence of the development of bladder cancer is known to be higher in industrialised societies [1].

The agents such as valrubicin, gemstabin, docataxel, cabazitaxel, mitomycin C, cisplatin and their combinations are used during the chemotherapeutic treatment of different subgroups of bladder cancer [2]. The use of cisplatin can

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provide different results due to the genetic differences of patients [3].

Cisplatin damages the tumor through apoptosis by attaching to the DNA and transduction pathways (calcium signaling, receptor signaling, activation of mitochondrial pathways, etc.). However, the most important problems in the use of cisplatin are the high systemic side effects experienced by the patients and the development of drug resistance and the decrease in the effectiveness of the treatment [4]. Various side effects associated with the use of chemotherapeutics are the undesirable systemic effects [5, 6]. Developed optimal treatment strategies may provide an advantage in reducing the side effects associated with the use of chemotherapeutics.

Researchers suggest that the administration of the active form of vitamin D calcitriol (1,25-dihydroxyvitamin D3 (1,25D3)), in combination with chemotherapeutic treatment may provide advantage for more effectively treatment of bladder cancer [7, 8]. Calcitriol has extensive regulatory effects on the regulation of cell metabolism including antiproliferation, proapoptotic effects, anti-cell migration and anti-angiogenic activities [9]. which changes the antitumoral activities of chemotherapeutic agents by several mechanisms such as cisplatin [10, 11] Various studies showed that calcitriol can support cisplatin-based chemotherapy [12] and have an anti-tumor effect in different types of cancer [13–15].

The ATP-binding cassette protein P-glycoprotein (P-gp) is an efflux pump and a product of the Multi Drug Resistance 1 gene (MDR1). P-gp is also frequently associated with multidrug resistance in human cancers [16, 17]. The P-gp is known to contribute to the absorption, distribution, metabolism and excretion of many clinically applied drugs. P-gp can reduce the absorption and oral bioavailability of drugs [17]. Considering that chemotherapeutic drugs are used in combination, it should be born in mind that the changes in P-gp expression level may play a role in response to treatment in bladder cancer.

Therefore, we aimed to evaluate the possible cytotoxic, apoptotic and P-gp expression level changes of calcitriol and cisplatin combination therapy on bladder cancer cell lines in vitro in our study.

Materials and methods

Chemicals and reagents

Stock solution was prepared by dissolving the calcitriol (Sigma Aldrich, USA) in ethanol at a concentration of 10,000 nM. Cisplatin (Kocak Farma, Turkey) is available in commercially purchased form as 25 mg/50 mL. Dilutions of all concentrations applied in the experiments were prepared using the cell culture medium.

Cell culture

T24 (human bladder carcinoma) and ECV-304 (human urinary bladder carcinoma) as bladder cancer cell lines and HUVEC (Human umbilical vein endothelial cells) as healthy control cell line from ATCC (American Type Culture Collection) were used in the study. Cell culture was performed using the Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma Aldrich, USA) in the presence of 10% fetal bovine serum (Biosera, France), 1% penicillin (100 U/mL)/ streptomycin (100 µg/mL)(Gibco, USA) in a humidified atmosphere containing 5% CO_2, 37 °C.

Experimental groups

T24, ECV-304 and HUVEC cell lines were divided into triplicated groups of 24, 48 and 72 h by treatment with different doses of calcitriol (25 nM, 50 nM, 100 nM, 500 nM, 1000 nM) after 24 h of culture for MTT analysis. MTT analysis of cisplatin, doses of 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml doses were treated in the cell cultures for 24, 48 and 72 h. All experimental groups were studied in three groups. 7.6 µg/ml (25.23 µM) used for cisplatin is stated in the literature as 100% Test Drug Concentration (%TDC) [18]. A level of 100% TDC is the approximate level of plasma peak concentrations of the chemotherapeutics [19]. In our study, when the 7.6 µg/ml (25.23 µM) dose of cisplatin was referenced as 100% TDC, the 5 µg/ml dose was specified as 66% TDC (16.6 µM) with percentage calculation. In the next process for Annexin V-PI flow cytometry and P-gp expression analysis, 24-h triplicated culture groups for were divided into groups for calcitriol 500 nM, cisplatin 5 µg/ml and combination treatment (calcitriol 500 nM + cisplatin 5 µg/ml) for each three cell lines.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed with slight modifications as previously described [20].Cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in 100 µL of culture medium and were incubated for 24 h to ensure the adherence. The cells were treated for 24 h, 48 h, 72 h with different concentrations of calcitriol (25–1000 nM), and cisplatin (16.6–166 µM). Combination treatments of the calcitriol (500 nM) and cisplatin (16.6 µM) were also performed in the same time periods. Triplicated applications were done for all samples. At the end of the treatment period, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. Following incubation for 4 h at 37 °C to allow the conversion of MTT to formazan

crystals, 100 µL of dimethyl sulfoxide (DMSO) was added and the formed formazan crystals were dissolved. The OD was measured at 570 nm using the ELISA microplate reader (Rayto RT-2100C,China). Viability of treated cells was calculated in reference to the untreated control cells using the following formula: Viability (%) = (100x(sample Abs)/(control Abs)), where Abs is the absorbance value at 570 nm.

Apoptosis analysis

For apoptosis assays, cells were seeded at a density of 5×10^5 cells per well of a six well plates. The cells were incubated for 24 h with calcitriol, cisplatin and combination treatments. Triplicated applications were done for all samples. At the end of the period, the cells were removed with trypsin, harvested, resuspended in cold PBS (to a concentration of 1×10^6 cells/mL) after centrifugation. After washing 2 times with PBS, Annexin V and PI dye staining process started. Samples were prepared prior to flow cytometric analysis according to the manufacturer's instructions (EXBIO Apo FlowEx FITC Czech Republic). The analysis of flow cytometry was carried out using Novocyte (Acea Biosciences, Inc.,USA).

P-glycoprotein analysis

P-glycoprotein is an important drug transporter that has been identified in various types of cancers and is associated with drug resistance. For the analysis of P-gp levels, cells treated with calcitriol, cisplatin or combination were trypsinized, harvested and centrifuged after 24 h. Cell pellets obtained by centrifugation were resuspended with cold PBS. Washing with PBS was repeated twice. Cells were prepared for staining of P-gp protein. For flow cytometric examination of P-gp expression, MDR1 (UIC2) conjugated to phycoerythrin was used (MDR-1 PE, Santa Cruz, USA). Cells to be analyzed were adjusted to 1×10^6 cells/mL. The study was performed using 1 µg of MDR-1 antibody per 1 million cells according to the manufacturer's instructions. All treatments were analyzed in triplicates. We used unstained control samples for each experimental group to evaluate the control samples and the drug-treated samples.

Statistical evaluation

The MTT, P-gp and Annexin V-PI values were statistically evaluated with the unpaired Student-T test using GraphPad Prism 5.04 Software. The groups were compared using the one-way analysis of variance (ANOVA). Tukey' s posthoc test was used for multiple comparisons due to homogeneity of variants. Correlation of MTT, apoptosis and P-gp results was evaluated by Pearson' s correlation analysis. $P < 0.05$ was considered as the significance value. Apoptosis and P-gp expression was analysed using the NovoExpress 1.3.0 software by flow cytometry.

Results

Optimal cytotoxic dose determination By MTT assay

Cytotoxic evaluation for determination of the optimum dose for calcitriol and cisplatin in T24, ECV-304, HUVEC cell lines was performed using the MTT test. The cytotoxic effect of 25–1000 nM doses for calcitriol, 16.6–166 µM doses for cisplatin were evaluated in all cells at 24 h, 48 h, 72 h (Fig. 1A–F). As a result of MTT, two different anti-proliferative doses (25 nM and 500 nM) were determined for calcitriol.

7.6 µg/ml (25.23 µM) cisplatin dose was referenced for 100% TDC, the 5 µg/ml dose was specified as 66% TDC (16.6 µM) with percentage calculation. The lower dose of cisplatin (16.6 µM; 66% TDC) was evaluated by MTT and showed cytotoxic activity. As a result of MTT experiments, the evaluations for calcitriol showed that the 500 nM dose was found as optimum for combination treatment for 24 h. In terms of cytotoxicity, the results revealed that calcitriol has a biphasic effect and a dose-dependent selective effect. According to the results, the combination treatment was maintained with 500 nM for calcitriol with 16.6 µM for cisplatin Fig. 1. Data showed that combination treatment increased the anti-proliferative efficacy compared to cisplatin alone ($p = 0.0108$) in T24 cells. Also it reduced the cytotoxicity in the HUVEC compared to cisplatin alone ($p < 0.0001$) (Fig. 1G). No change in combination treatment increased the anti-proliferative efficacy compared to cisplatin alone in ECV-304 cell line ($p = 0.5778$).

Apoptosis and P-gp expression results

Apoptosis which was applied with Annexin V/PI staining, evaluated for 500 nM calcitriol, 16.6 µM cisplatin, and combination treatment (500 nM calcitriol + 16.6 µM cisplatin) for 24 h. Our results showed that the combination treatment achieved a higher apoptosis rate in T24 cell line than the rate of cisplatin treatment alone ($p = 0.0005$). It was determined that the rate of apoptosis showed no significant change in the ECV-304 cell line. The rate of apoptosis was found to have decreased in HUVEC healthy control cell line ($p = 0.0082$) (Fig. 2).

P-gp expression levels were evaluated in the presence of either 500 nM calcitriol, or 16.6 µM cisplatin, or in the presence of both 500 nM calcitriol and 16.6 µM cisplatin (combined treatment) for 24 h. P-gp levels were found to be higher in the T24 cell line following combined treatment (500 nM calcitriol + 16.6 µM cisplatin; $p < 0.0001$) than in

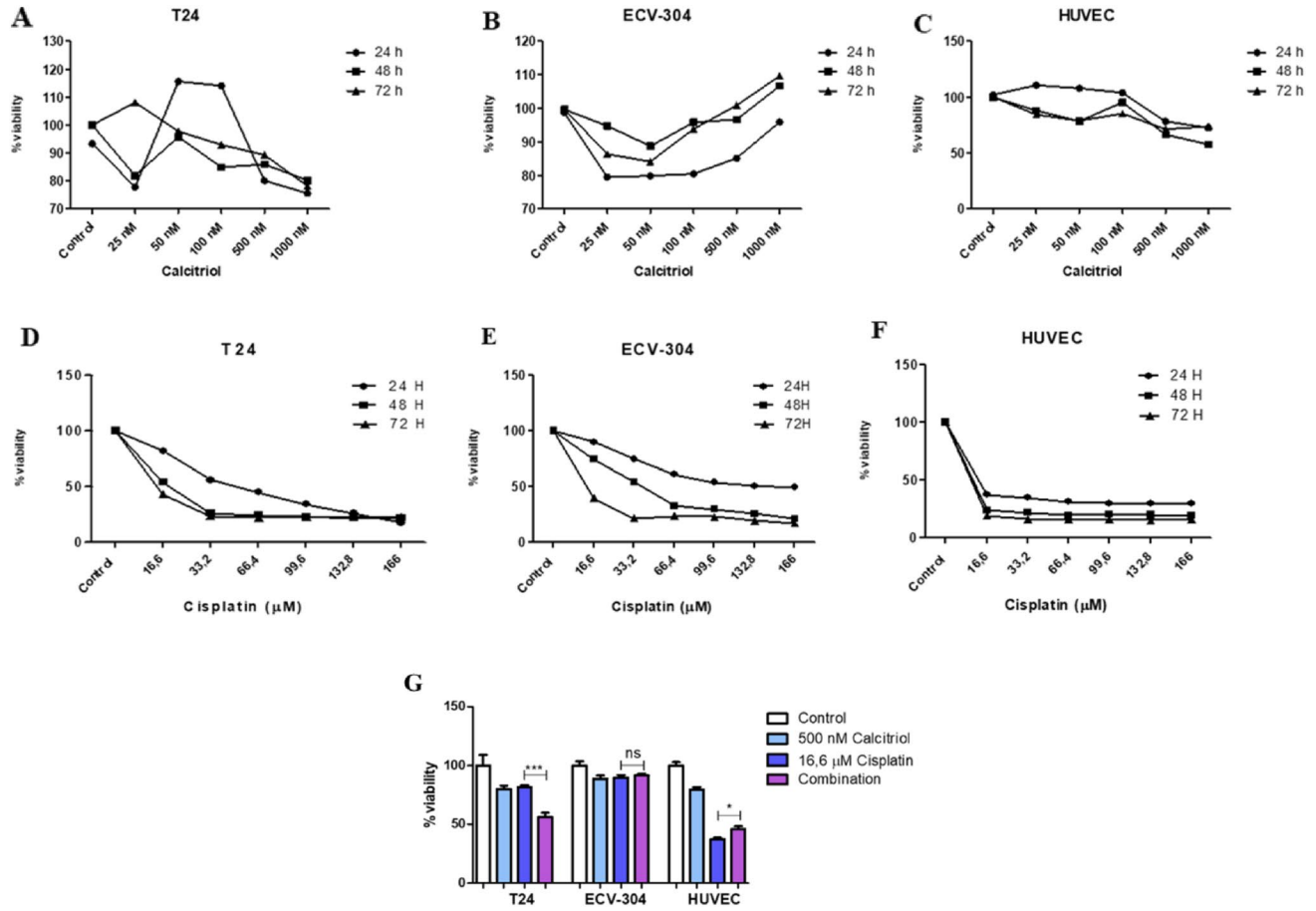


Fig. 1 MTT assay results for calcitriol treatment in T24 (**A**), ECV-304 (**B**), HUVEC (**C**) cell lines with doses between 25 to 1000 nM for 24 h, 48 h and 72 h. Cisplatin treatment in T24 (**D**), ECV-304 (**E**), HUVEC (**F**) cell lines with doses between 16.6 μM to 166 μM for 24 h, 48 h and 72 h. Cells treated with cisplatin after 24 h cell cultivation. (**G**); Comparison of MTT results. Control cells (non treated), 500 nM calcitriol, 16.6 μM cisplatin and 500 nM calcitriol + 16.6 μM cisplatin for combination treatment was evaluated for 24 h. Triplicated applications were done for all samples. Data are mean ± SEM (n = 3), (***p < 0.001, **p < 0.01, *p < 0.05, ns: non significant)

the cisplatin alone or calcitriol alone treatments (Table 1; Fig. 3).

In addition, no significant changes were observed in terms of P-gp expression rates in the ECV-304 cell line. The statistical evaluation of the cisplatin treatment compared to the combination treatment in the ECV-304 cell line showed that, the significance value was p = 0.0616 (Table 1; Fig. 3).

The expression levels of P-gp in HUVEC cell line increased with calcitriol treatment compared to the level in the control p < 0.0001, while decreased significantly with cisplatin treatment compared to the level in the control (p = 0.0005). P-gp expression levels in HUVEC cells increased in combination treatment compared to the levels in the control and cisplatin alone (p < 0.0001) (Table 1; Fig. 3).

Calcitriol treatment showed positive correlation between apoptosis and P-gp results in T24 cell line. In the combination treatment, negative correlation was determined between MTT and P-gp results in both ECV-304(p = 0.0033, r = −0.9524) and HUVEC cell lines(p < 0.0001, r = −0.9988) A strong positive correlation was shown between MTT and P-gp results in cisplatin treatment in HUVEC cells(p = 0.0016, r = 0.9676) (Table 2).

Discussion

Cisplatin is a key ingredient for chemotherapeutic therapy in bladder cancer as part of drug combinations with methotrexate, vinblastine, adriamycin and cisplatin (MVAC) or gemcitabine, cisplatin (Gem–Cis) used to treat urethelial cell carcinomas [21–23].

It is known that side effects can occur as a result of chemotherapeutic drug use. Reducing the systemic side effects is extremely important in the success of bladder cancer treatment [24]. There are studies in squamous cell carcinoma which support our study of the use of calcitriol in combination with cisplatin. These studies provide preclinical data

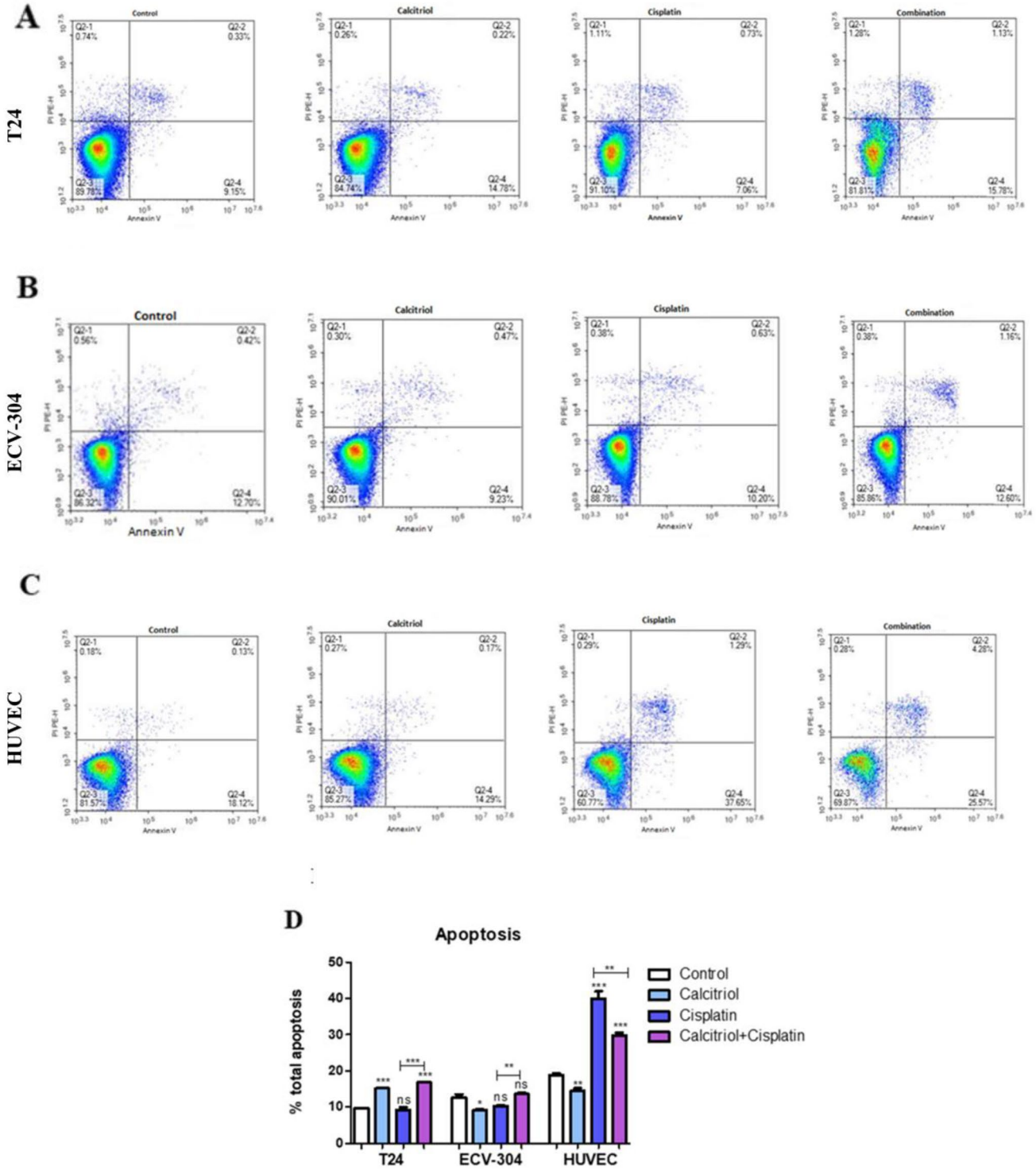


Fig. 2 Annexin V/PI staining represented images for T24 (**A**), ECV-304 (**B**) and HUVEC (**C**) cell lines for 24 h. Control cells (non treated), 500 nM calcitriol, 16.6 µM cisplatin and 500 nM calcitriol + 16.6 µM cisplatin for combination treatment was evaluated. Total apoptosis results for T24, ECV-304 and HUVEC (**D**).All experiments were repeated three times. Data are mean ± SEM (n = 3), (***p < 0.001, **p < 0.01, *p < 0.05, ns: non significant)

Table 1 Percentage ratios of MFI (Mean Fluorescence Intensity) obtained from flow cytometric analysis of P-gp expressions levels in T24, ECV-304 and HUVEC cells

Cell lines	Control	Calcitriol (500 nM)	Cisplatin (16.6 µM)	Calcitriol + Cisplatin (500 + 16.6 µM)
T24	74.47	79.22	80.65	68.33
ECV-304	97.64	99.18	98.05	97.47
HUVEC	87.08	92.50	86.09	92.16

Data are mean ± SEM (n = 3). The difference between the treatments was analysed using the unpaired student's T test

indicating that the combination of these two molecules enhances apoptosis and anti-tumoral response [25, 26]

One study stated that calcitriol changed the antitumoral response in pancreatic cancer models with one of the other chemotherapeutic drugs of gemcitabine together with cisplatin [27].

In another study, Konety et al. [28], evaluated doses between 0.8 µM and 10 µM for calcitriol in T24 and 253j cell lines and observed that proliferation was strongly inhibited. The IC50 dose specified in this study for calcitriol is 40 µM. We used 500 nM calcitriol in our experiments, in which we could benefit from the antiproliferative effect at the maximum level from the groups in our dose range.

The T24 cells were treated with calcitriol for 24 h and then with cisplatin for 48 h in the study conducted by Bunch et al. [25]. Researchers stated that 1,25D3 increases the response to cisplatin due to Vitamin D receptor in vitro. Control groups and cell lines treated with calcitriol showed different responses in terms of apoptotic association. In this study T24 cells were treated with EtOH, 100 nM of 1,25D3, 0.1 µg/mL of cisplatin, or the combination. In contrast, we evaluated 500 nM calcitriol, 16.6 µM cisplatin and 500 nM calcitriol + 16.6 µM cisplatin for combination therapy with MTT for 24 h.

In a study conducted by Ma et al. [10], 1,25D3 (calcitriol) enhances gemcitabine and cisplatin-induced apoptosis. T24 cells were treated with EtOH or 1.5 µM 1,25D3 for 24 h, followed by 25 nM gemcitabine and 0.75 µg/mL cisplatin for 48 h. T24 cells expressed no detectable Vitamin D receptor (VDR) and calcitriol induced VDR expression in a dose dependent manner. Calcitriol alone did not induce apoptosis in T24. Apoptosis was slightly increased by the combination of gemcitabine and cisplatin, which was further significantly increased by the pretreatment with calcitriol. Although there are differences in treatment in our study compared to this study, it gives clues that calcitriol changes the efficacy of cisplatin.

We started our experiments using the bladder cancer-associated ECV-304 cell line with the resources of our laboratory. The ECV-304 cell line was previously identified as a bladder cancer-associated cell line [29, 30]. However, researchers have also defined in the literature that the ECV-304 cell line is a transformed and crosscontaminated cell line from the HUVEC cell line [31, 32]. Brown et al. [33] showed that ECV-304 is genetically similar to the bladder cancer cell line T24/83. ECV-304 has been identified as a controversial cell line in studies, and therefore we added the T24 cell line to our experiments in the later stages of our study. Thus, at the same time these two cell lines were compared in terms of their responses to our experiments. In our study, we found different values in cytotoxic, apoptotic and P-gp expression results of cisplatin and calcitriol treatment to T24, ECV-304 and HUVEC cell lines.

While calcitriol treatment gave different responses in different cell lines, it could also give variable responses depending on the dose (Fig. 1). Some lower doses of calcitriol used in our experiments had a higher antiproliferative effect, while higher dose treatment could have a lower antiproliferative effect (Fig. 1A–C) which we named as the biphasic effect of calcitriol. Therefore we suggest that studies with different doses and different cell lines are needed and should be careful in the use of vitamin D. It should also be considered that the use of calcitriol in high doses may cause hypercalcemic side effects in individuals [34].

We suggest that the regulating effect of calcitriol should be used more than the anti-proliferative effect of calcitriol. The data in our study show that calcitriol can be used as an adjunct to reduce the cisplatin dose in use or to increase the efficiency of administration. The reduction in apoptosis level in HUVEC healthy cell line and the increase in apoptosis level in T24 bladder cancer cell line with combination treatment are promising. However, although the decrease in the apoptotic level of healthy cells is a valuable data, further studies should be done in terms of genomic instability when the effects of cisplatin are considered. Our study is at the preclinical level and needs to be supported by further studies for clinical use.

The P-gp molecule is frequently associated with multidrug resistance in human cancer [16, 35]. In our study, we aimed to evaluate the P-gp expression that may be affected by calcitriol and cisplatin combination treatment. Various publications in the literature investigated the interaction of P-gp molecule. Tada et al. showed that the overexpression of the P-gp gene is closely associated with the clinical outcome of various malignancies and plays a role in the response to the use of chemotherapeutic agents [36]. P-gp, which acts as an efflux pump, actively reduces the intracellular accumulation of anticancer drugs and in the last decade, the clinical significance of P-gp expression levels rather than other relevant ATP-binding cassette (ABC) transporters in poor chemotherapy treatment prognosis has also received much attention [37]. However Massari et al. stated that P-gp expression is not associated with cisplatin-based

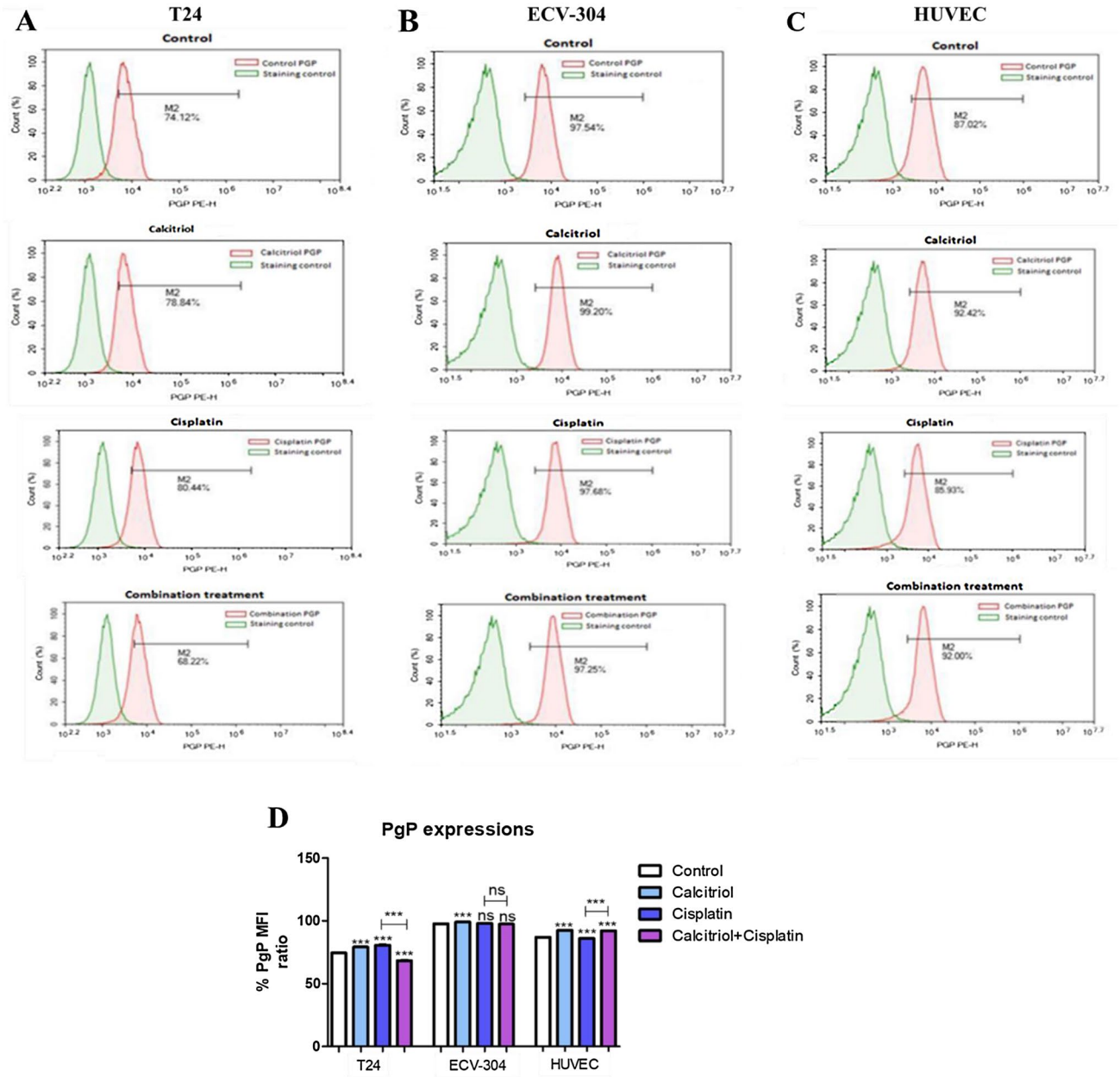


Fig. 3 Represented images of P-gp exspression for T24 (**A**), ECV-304 (**B**) and HUVEC (**C**) cell lines for 24 h. P-gp results evaluation of control cells (non treated), calcitriol (500 nM), cisplatin (16.6 μM) and combination treatment (Calcitriol 500 nM + cisplatin 16.6 μM) for each three cell line (**D**). All experiments were repeated three times. Data are mean ± SEM (n = 3), (***p < 0.001, **p < 0.01, *p < 0.05, ns: non significant)

chemotherapy response [22]. In contrast Sun et al. [38] defined that the prolonged time of cisplatin treatment induces elevated expression of P-gp in T24 cell line.

Researchers stated in the literature that cisplatin is not a substrate of P-gp [39]. However, the combination of cisplatin and calcitriol caused a change in the level of P-gp in the T24 cell line in our experiments. Our data showed that the combination treatment caused a decrease in P-gp in T24 bladder cancer cells and an increase in P-gp levels in HUVEC healthy cell line (Fig. 3; Table 1). A decrease in the apoptosis rate was detected in the HUVEC healthy cell line (Fig. 2) with the increase of P-gp (Table 1). In the interpretation of our data, we suggest that the change in P-gp levels is not only associated with cisplatin treatment, but with the help of combination treatment containing calcitriol. Our data suggest that combination treatment can be used as an advantage in conditions that

Table 2 The correlation analyzes between MTT, apoptosis and P-gp data corresponding to calcitriol, cisplatin and combination treatments in T24, ECV-304 and HUVEC cell lines

	T24 cell line		ECV-304 cell line		HUVEC cell line	
	Apoptosis	P-gp	Apoptosis	P-gp	Apoptosis	P-gp
Calcitriol						
MTT	0.9236 (ns)	0.6829 (ns)	0.9072 (ns)	0.2863 (ns)	0.7119 (ns)	0.6486 (ns)
Apoptosis		0.0314 (*) r = 0.8517		0.9256 (ns)		0.6990 (ns)
Cisplatin						
MTT	0.8541 (ns)	0.6670 (ns)	0.6325 (ns)	0.5219 (ns)	0.4157 (ns)	0.0016 (**) r = 0.9676
Apoptosis		0.3857 (ns)		0.0799 (ns)		0.7481 (ns)
Cal + Cis						
MTT	0.5756 (ns)	0.3105 (ns)	0.3353 (ns)	0.0033 (**) r = −0.9524	0.7071 (ns)	< 0,0001 (***) r = −0.9988
Apoptosis		0.1361 (ns)		0.7186 (ns)		0.6396 (ns)

The p values of the correlation data are given in the table. $P < 0.05$ was considered as the significance value. Ns = not significant, r = Pearson's r value ($r > 0.8$ represents very high correlations.) Pearson's correlation analyzes were performed to assess statistical significance

affect the treatment due to P-gp. In addition, our data contributes to these studies about P-gp. It gave us possible clues that combination treatment may also help to reduce the dose of chemotherapeutic combinations that are used for the bladder cancer treatment.

Our study reveals that calcitriol preclinically increases the effectiveness of cisplatin in bladder cancer cells. It also provides data that can be considered in the development of viable pharmacokinetic models. Considering the side effects of cisplatin, revealing the data to be obtained from studies on combination treatments with important regulatory molecules such as vitamin D may enable to increase the survival rate and quality of life for bladder cancer patients.

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Author contributions OO, OEG, KS.,YI conceived and designed the present study. OO and OEG performed the experiments. OO and OEG was responsible for the data analysis and performed data interpretation. OO, OEG, KS, YIHC, KO wrote the paper, revised the manuscript. AN is responsible for the flow cytometric analysis.

Data availability The datasets supporting the conclusions of this article are included within the article.

Declarations

Conflict of interest The authors have declared that they have no competing interests. Ethical approval not applicable to the approval.

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