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Research Article

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

Posted Date: March 2nd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1389662/v1>

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Does the regulatory effect of vitamin D allow to reduce the cisplatin dose in T24 bladder cancer cell line?

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Abstract

1,25(OH)₂D₃ (Calcitriol, active form of Vitamin D), which is a broad regulatory molecule, plays a role in changing the efficacy of chemotherapeutic drugs. In bladder cancer, effectiveness of the treatment and reducing the resistance to chemotherapeutics such as cisplatin are of great importance. Cisplatin usage is one of a current standard chemotherapy regimen for bladder cancer. In our study T24, ECV-304 and HUVEC cell lines were treated with calcitriol and cisplatin individually and with combination. MTT assays were used for optimal dose determination. It was aimed to determine the possible potential of the combined use of cisplatin with calcitriol. Determined doses and combination treatments of calcitriol and cisplatin on all cell lines were evaluated for cytotoxicity, apoptosis and drug resistance. Also calcitriol and cisplatin interaction with p53 protein was assessed by molecular docking analysis. Datas 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). Combination treatment achieved slightly higher apoptosis rate in T24 cells than treatment of cisplatin alone (p=0.0005). It was determined that the rate of apoptosis and P-gp levels did not change significantly in the ECV-304 cells. However it decreased in HUVEC healthy control cell line (p=0.0082). P-gp ratios were increased in HUVEC (p < 0.0001) and decreased in T24 cells (74.47% p<0.0001) compare to control. The data obtained in this study show that the combination of calcitriol and cisplatin allows the use of cisplatin at lower doses.

Key Words: Bladder cancer, T24 cell line, Calcitriol, Cisplatin, Vitamin D, P-gp, Apoptosis

Introduction

Variations in multiple cancer rates, 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 in worldwide. Its incidence in men is three times higher than in women. It is known that environmental, demographic and genetic factors are effective in the development of bladder cancer. Although there are various difficulties in determining the rates of effect on disease development, it is known that the incidence of bladder cancer development is high in industrial societies (1).

During the chemotherapeutic treatment of bladder cancer, agents such as valrubicin, gemstabin, docataxel, cabazitaxel, mitomycin C, cisplatin and their combinations are used (2). Various side effects associated with the

use of these chemotherapeutics are undesirable systemic effects. Developed optimal treatment strategies may provide an advantage in reducing the side effects associated with chemotherapeutic usage.

In addition to chemotherapeutic treatment, calcitriol (1,25-dihydroxyvitamin D₃ (1,25D₃)) the active form of vitamin D, may also be an option for the development of more effective treatment for bladder cancer (3-4). Calcitriol has several beneficial effects about the regulation of cell metabolism including anti-proliferation, pro-apoptotic effects, anti-cell migration and anti-angiogenic activities (5).

The pathogenesis of bladder cancer emerges with mutations in somatic cells and failure of protooncogenes and tumor suppressor genes to maintain their normal functions (6). P53 is one of the most commonly mutated tumor suppressor gene in cancer. P53 loss is associated with a variety of undesirable phenotypic properties, including apoptotic signaling error and lack of cell cycle checkpoint regulation (7). Mutations in p53 are associated with cisplatin resistance in many cancer types and poor prognosis in bladder cancer (8-9).

Cisplatin damages the tumor through apoptosis by connecting with 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 (10).

Therefore, in our study, we aimed to evaluate the possible cytotoxic, apoptotic and drug resistance effects of combination therapy on bladder cancer cell lines in vitro.

Materials and Methods

Chemicals and reagents

Stock solution was prepared by dissolving calcitriol (Sigma) in ethanol at a concentration of 10000 nM. Cisplatin (Kocak Farma) is available in commercially purchased form as 25 mg/50 mL. Dilutions of all concentrations applied in the experiments were prepared using cell culture medium.

Cell Culture

The T24 human bladder cancer cell line, ECV-304 cancer cell line and HUVEC endothelial cell line from ATCC (American Type Culture Collection) for the control cell line were cultured for our studies. Cell culture was performed using Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma Aldrich) in the presence of 10% fetal bovine serum (Biosera), 1% penicillin (100 U/mL)/ streptomycin (100 µg/mL) (Gibco) in a humidified atmosphere containing 5% CO₂.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed as previously described. 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 24h, 48h, 72h with each 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 at the same time periods. In brief, 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 formazan crystals formed were dissolved. The OD was measured at 570 nm using ELISA microplate reader (Rayto RT-2100C, AB Medical). Viability of treated cells was calculated in reference to the untreated control cells using the following formula: $\text{viability (\%)} = (100 \times (\text{sample Abs}) / (\text{control Abs}))$, where Abs is the absorbance value at 570 nm (11).

Analysis of Apoptosis

The cells were incubated for 24 h with calcitriol, cisplatin and combination treatments in 6-well plates. 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. Sample preparations prior to flow cytometric analysis were performed according to the manufacturer's instructions (EXBIO Apo FlowEx FITC). 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 cancer and is well known to be associated with chemotherapy resistance.

Cells treated with calcitriol, cisplatin and combination were trypsinized, harvested and centrifuged after 24 hours. Cell pellets obtained by centrifugation were resuspended with cold PBS. Washing with PBS was repeated 2 times. Cells were prepared for staining of P-gp protein. For flow cytometric examination of P-gp expression, MDR1 (UIC2) was used conjugated to phycoerythrin (MDR-1 PE, Santa Cruz). Cells to be analyzed were adjusted to 1×10^6 cells/mL. Staining and analysis of P-gp protein was performed according to the manufacturer's instructions.

Docking Analysis

In the molecular docking study, the activities of calcitriol and cisplatin molecules against p53 protein were investigated. Hex 8.0.0 (a well-known highly advanced protein and ligand docking tool) was used for protein and drug interaction. Docking data was calculated using the HEX 8.0.0 program for this protein docking study.

The structure of p53 was retrieved from protein data bank (PDB) archive as PDB ID: 2MWY.

Statistical Evaluation

MTT, P-gp and Annexin V-PI values were statistically evaluated with the unpaired Student-T test using GraphPad Prism 5.04 Software. HEX 8.0.0 program was used in the evaluation of molecular docking datas. Analysis of apoptosis and P-gp expression by flow cytometry was performed with NovoExpress 1.3.0 software.

Results

Optimal Cytotoxic Dosage Determination By MTT Assay

Cytotoxic evaluation for determination of optimum dose for calcitriol and cisplatin in T24, ECV-304, HUVEC cell lines was performed by MTT test. The cytotoxic effect of 25nM -1000 nM doses for calcitriol, 16.6 μ M - 166 μ M doses for cisplatin was evaluated in all cells at 24h, 48h, 72h. As a result of MTT, two different anti-proliferative doses (25nM and 500nM) were determined for calcitriol. The optimum dose of cisplatin 7.6 μ g/ml for 100% TDC (25.23 μ M) was reported in the literature (12).

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

The lower dose of cisplatin (16.6 μ M; 66% TDC) was evaluated by MTT and showed cytotoxic activity. As a result of MTT experiments, when all three cell lines were evaluated for calcitriol, 500 nM dose was found to be 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 μ M for cisplatin (Fig. 1).

Apoptosis and P-gp Expression Results

Apoptosis which was applied with Annexin V/PI staining, evaluated for 500nM calcitriol, 16.6 μ M cisplatin, and combination treatment (500nM calcitriol +16.6 μ M cisplatin) for 24 h. According to our results, combination treatment achieved a higher apoptosis rate in T24 cell line than treatment of cisplatin alone (p=0.0005). It was determined that the rate of apoptosis did not change significantly in the ECV-304 cell line. It was observed that the rate of apoptosis decreased in HUVEC healthy control cell line (p=0.0082) (Fig. 2).

Examination of P-gp expression level evaluated for 500nM calcitriol, 16.6 μ M cisplatin, and combination treatment (500nM calcitriol +16.6 μ M cisplatin) for 24 h. We found that the P-gp expression level of the combination treatment in the T24 cell line decreased by $p<0.0001$ compared to the control and $p<0.0001$ compared to the treatment of only cisplatin and only calcitriol (**Fig. 3**).

In the HUVEC cell line, the expression rate of P-gp increased with calcitriol treatment compared to the control $p<0.0001$, while it decreased significantly with cisplatin treatment compared to the control ($p=0.0005$). P-gp expression levels in HUVEC cells, increased in combination treatment compared to control $p<0.0001$. In addition, no significant changes were observed in terms of P-gp expression rates in the ECV-304 cell line. When the cisplatin treatment was evaluated statistically compared to the combination treatment in the ECV-304 cell line, the significance value was $p=0.0616$ (**Table 1, Fig.3**).

Fig 2 Annexin V/PI staining represented images for T24 (**A**), ECV-304 (**B**) and HUVEC (**C**) cell lines for 24h. Control cells (non treated), 500 nM calcitriol, 16.6 μ M cisplatin and 500 nM calcitriol +16.6 μ M cisplatin for combination treatment was evaluated. (**D**) Total apoptosis results for T24, ECV-304 and HUVEC. Triplicated applications were done for all samples. Data are mean $\pm$ SEM (n = 3), (** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns: non significant).

Fig 3 Represented images of P-gp expssion for T24 (**A**), ECV-304 (**B**) and HUVEC (**C**) cell lines for 24h. (**D**) P-gp results evaluation of control cells (non treated), calcitriol (500 nM), cisplatin (16.6 μ M) and combination treatment (Calcitriol 500nM+ cisplatin 16.6 μ M) for each three cell line. Triplicated applications were done for all samples. Data are mean $\pm$ 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.

<i>Cell lines</i>	<i>Control</i>	<i>Calcitriol (500nM)</i>	<i>Cisplatin (16.6 μM)</i>	<i>Calcitriol+ Cisplatin (500nM+ 16.6 μM)</i>
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 $\pm$ SEM (n = 3). The difference between the treatments was analyzed by unpaired student's t test.

Docking Analysis

According to docking analysis, calcitriol has higher binding energy with p53 molecule compared to cisplatin. It was evaluated simulatively with DNA Docking method in order to form the basis for future research.

Fig 4 Calcitriol-p53 and cisplatin-p53 interaction structures via to Docking Analysis.

Table 2 Docking analysis results showing the binding energies of calcitriol and cisplatin molecules in their interaction with p53 protein.

	<i>E_{total}</i>	<i>E_{shape}</i>	<i>E_{force}</i>
Calcitriol	-282.10	-282.10	0.00
Cisplatin	-135.16	-135.16	0.00

Docking data was calculated using the HEX 8.0.0 program for this protein docking study.

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 (13). It is known that side effects can occur as a result of chemotherapeutic drug usage. Reducing systemic side effects is extremely important in the success of bladder cancer treatment. In our study, the combined use of calcitriol molecule, which has high regulatory functions, with cisplatin was evaluated in terms of anti-proliferative, apoptotic properties and drug resistance in bladder cancer cell lines.

One of the cell lines we evaluated, the ECV-304 cell line, was identified as a bladder cancer-associated cell line in the past years. However, ECV-304 is thought to be transformed from the HUVEC cell line in the literature (14,15). In addition, there is also a study showing the genetic identification of ECV-304 with the bladder cancer cell line T24/83 (16). In our study, we determined different values in cytotoxic, apoptotic and P-gp expression results of T24, ECV-304 and HUVEC cell lines for cisplatin and calcitriol. In this way, three different cell lines were compared with each other.

In our experiments, we adjusted the optimum dose of cisplatin and calcitriol to remain below the 100% TDC (Test Drug Concentration) level. A level of 100% TDC is the approximate level of plasma peak concentrations of the chemotherapeutics (12). Because exceeding 100% TDC doses could come out systemic side effects when it comes to use in advanced clinical applications.

The 100% TDC dose of cisplatin in the literature was (25.23 μ M) (12). Therefore, we chose the ratio below the 100% TDC dose and 66% TDC (16.6 μ M) as the combination dose for cisplatin in our experiments. According to the literature, the IC₅₀ dose for calcitriol in T24 bladder cancer cells was 40 μ M (17). Based on our MTT data on calcitriol, we benefited from its anti-proliferative properties at much lower dose levels (25nM-500nM) of calcitriol. We have determined that calcitriol has a biphasic effect at the cellular level. Calcitriol induced dose-dependent variable responses (**Fig. 1**). According to our basic approach, could we use the effect of calcitriol to reduce the dose of cisplatin or achieve the same effectiveness at a lower dose? As a preclinical data, it seems that yes we can.

There are studies in squamous cell carcinoma and pancreatic models that support our study of the use of calcitriol in combination with cisplatin. These studies provide preclinical data indicating that the combination of these two molecules enhances apoptosis and anti-tumoral response (18,19,20). In another study, it was done with the doses between 0.8 and 10 μ M for calcitriol in T24 and 253j cell lines observed that proliferation was strongly inhibited. They mention that normal and neoplastic bladder tissue and cell lines express the Vitamin D receptor. Control groups and cell lines treated with calcitriol showed different responses in terms of apoptotic association. The results of this study gave data that calcitriol may be associated with bladder cancer (17, 18). However, studies on calcitriol and bladder cancer are limited.

Calcitriol has extensive regulatory effects. It changes the antitumoral activities of chemotherapeutic agents such as cisplatin by several mechanisms (8,21). There are various publications showing that calcitriol can support cisplatin-based chemotherapy and have an anti-tumor effect in different types of cancer (22, 23, 24).

The P-gp molecule is associated with drug resistance in the cell. In our study, we wanted to evaluate the P-gp expression that may be caused by calcitriol and cisplatin. There are various publications in the literature about the interaction of cisplatin and P-gp with each other. Tumors from patients treated with MVAC have been found to express higher levels of P-gp compared to untreated patients (25-26). However, most studies of P-gp expression in urethral carcinoma cells (UCC) show that it is not associated with cisplatin-based chemotherapy response (27, 28, 29). A recent large study at UCC suggests that increased P-gp expression is associated with a poor response to MVAC, and this effect is also thought to be due to cisplatin (30, 31, 32). According to the in vitro studies producing doxorubicin-resistant UCC cells expressing high P-gp indicate that there is no increase in cisplatin resistance (28, 33, 34, 35). The role of P-gp in terms of overall clinical response and therapeutic function remains controversial. In addition, our data make an additional contribution to these studies about P-gp. According to our data, the increase in P-gp expression level was consistent with apoptosis levels and gave significant results. The combination treatment caused a decrease in P-gp in T24 bladder cancer cells and an increase in P-gp in HUVEC healthy cell line. It also advantageously changed the apoptosis levels (**Fig. 2, Fig. 3** and **Table 1**). With the increase of P-gp, a slight decrease in the apoptosis rate was detected in the HUVEC healthy cell line. These data gave us

possible clues that calcitriol may help to reduce the dose in the use of cisplatin, which has many side effects, and may contribute to the survival of healthy cells.

It is also shown in the literature that the T24 cell line has p53 mutation (36). Although we could not experimentally demonstrate the effectiveness of calcitriol and cisplatin against p53 protein, we wanted to evaluate it simulatively with DNA Docking method in order to form the basis for future research. At this point, it was started with the idea that p53 is one of the main targets of cisplatin and that changes in p53 may play a role in the response to chemotherapy. Sarkis et al. showed that p53 damage is a predictor of MVAC treatment success and survival ratios in patients with invasive bladder cancer (37). The p53 status is helpful in determining the response to chemotherapy or surgical procedures. Conversely, in the study of Cote et al., they found that the local and regional TCC patient group with abnormal p53 expression benefited from cisplatin-based adjuvant chemotherapy (38,39,40). According to the datas we have reached, it has been determined that calcitriol has a higher rate of interaction against the p53 protein than cisplatin (**Table 2, Fig. 4**).

Our study reveals that calcitriol increases the effectiveness of cisplatin in bladder cancer cells preclinically. 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 and life quality of bladder cancer patients.

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Acknowledgements

The present study was supported by a grant from the Scientific Research Projects Coordination Unit of Istanbul University (Doctorate Project no: TDK-2017-23523).

Thanks to Abdullah Yilmaz about flow cytometric analysis. Also we thanks to Assoc Prof Dr Nuray Varol for the material supplement.

Author contributions O.O.,O.E.G, K.S.,Y.I conceived and designed the present study. O.O. and O.E.G performed the experiments. O.O. and O.E.G was responsible for the data analysis and performed data interpretation. O.O.,O.E.G, K.S.,Y.I wrote the paper, revised the manuscript. A.N. is responsible for the flow cytometric analysis. H.C. is responsible for the docking 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 because this study was performed using cell lines.

Figures

Figure 1

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

Figure 2

Annexin V/PI staining represented images for T24 (A), ECV-304 (B) and HUVEC (C) cell lines for 24h. Control cells (non treated), 500 nM calcitriol, 16.6 μ M cisplatin and 500 nM calcitriol +16.6 μ M cisplatin for combination treatment was evaluated. (D) Total apoptosis results for T24, ECV-304 and HUVEC. Triplicated applications were done for all samples. Data are mean $\pm$ SEM (n = 3), (**p<0.01, *p<0.05, ns: non significant).

Figure 3

Represented images of P-gp exspression for T24 (A), ECV-304 (B) and HUVEC (C) cell lines for 24h. (D) P-gp results evaluation of control cells (non treated), calcitriol (500 nM), cisplatin (16.6 μ M) and combination treatment (Calcitriol 500nM+ cisplatin 16.6 μ M) for each three cell line. Triplicated applications were done for all samples. Data are mean $\pm$ SEM (n = 3), (**p<0.01, *p<0.05, ns: non significant).

Figure 4

Calcitriol-p53 and cisplatin-p53 interaction structures via to Docking Analysis.