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Calcitriol Combined With Platinum-based Chemotherapy Suppresses Growth and Expression of Vascular Endothelial Growth Factor of SKOV-3 Ovarian Cancer Cells

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Abstract. *Aim: To evaluate the effects of the combination of calcitriol and chemotherapy and to identify the molecular mechanisms underlying the effect of calcitriol on ovarian cancer cells. Materials and Methods: SKOV-3 cells were treated with calcitriol and cisplatin, and their effects alone and in combination in a dose-dependent manner were compared. Cell viability, cell proliferation, and apoptosis were assessed using the following assays: PrestoBlue, intracellular adenosine triphosphate, caspase-3/7 activity, annexin V, and immunoblotting, respectively. Results: Calcitriol alone caused dose-dependent inhibition of cell survival and proliferation, and induced apoptotic cell death of SKOV-3 cells. We confirmed that the expression of vitamin D receptor was increased in a dose-dependent manner by calcitriol. Combination treatment using calcitriol at a physiological concentration of 10-100 nM plus cisplatin significantly suppressed cell survival and induced apoptosis. Furthermore, when calcitriol was administered alone, the activity of vascular endothelial growth factor decreased in a dose-dependent manner, and when combined with cisplatin, activity was more suppressed. Conclusion: In SKOV-3 ovarian cancer cells, calcitriol plus cisplatin exerted greater antiproliferative, apoptotic, and anti-angiogenic effects than cisplatin alone. Adding calcitriol to platinum-based chemotherapy might be beneficial to patients with ovarian cancer.*

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Ovarian cancer is the main cause of mortality in women with cancer (1). The 5-year survival rate for women with epithelial ovarian cancer (EOC) is less than 40% because, in most cases, the disease is diagnosed at an advanced stage (2, 3). Patients with advanced EOC are treated with cytoreductive surgery and platinum-based chemotherapy; those who receive adjuvant chemotherapy or preoperative chemotherapy will also undergo cytoreductive surgery (2, 4). Patients with advanced EOC respond well to initial platinum-based therapy but the disease eventually recurs in more than 75% of patients (5). Patients with recurrent EOC receive additional chemotherapy, which increases the survival rate, but does not ultimately cure the disease. The treatment modalities for ovarian cancer have not significantly progressed over the past few decades, so there is growing interest in new approaches for EOC treatment (6). The growing biological understanding of EOC has led to the development of several targeted molecules and biological treatments, including angiogenesis inhibitors, immune modulators and poly (ADP-ribose) polymerase (PARP) inhibitors (7).

Vitamin D has a well-established role in maintaining calcium and bone homeostasis. Nearly 3% of the human genome is affected by the endocrine system of vitamin D (8). Calcitriol is an activated hormone of vitamin D that binds to the vitamin D receptor (VDR) in the cell nucleus (9, 10). Studies have reported mechanisms by which VDR can suppress tumor growth, including genomic and non-genomic signaling pathways (8, 10, 11). In addition, calcitriol regulates important biological actions, including insulin secretion, cell differentiation, apoptosis, proliferation, immune responses, angiogenesis, invasion and metastasis, and anti-inflammatory actions (9). Reduced exposure to the sun, and lack of vitamin D, are associated with increased cancer risk (12-15).

Many VDR target genes have been found in ovarian cancer cells (16). Furthermore, calcitriol has been found to facilitate cell apoptosis and cell-cycle arrest in ovarian

cancer cells (17-19). However, the main mechanisms involved in calcitriol activity in ovarian cancer have not been elucidated, and the effect of its combination with anticancer drugs has not widely been studied. Therefore, we aimed to identify the potential effects on ovarian cancer cells in vitro, and the mechanisms of action, of calcitriol, alone and in combination with cisplatin, a platinum-based anticancer drug that is at the core of ovarian cancer treatment.

Materials and Methods

Cell culture and drugs. We purchased the human ovarian cancer cell line SKOV-3 (Rockville, MD, USA). The SKOV-3 serous-type ovarian cancer cell line was cultured in McCoy's 5A medium (Welgene, Kyungsan, Republic of Korea) containing 10% 1% penicillin–streptomycin (P/S; Invitrogen, Carlsbad, CA, USA) and fetal bovine serum in an incubator at 37˚C with 5% CO_2. Calcitriol and cisplatin were obtained from Selleckchem (Houston, TX, USA), and the reagents were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA), the final concentration of which in the culture medium did not exceed 0.1%.

Cell viability. We assessed cell viability using PrestoBlue cell viability reagent (Invitrogen). SKOV-3 cells (1×10^5 cells/well) were plated in 96-well plates in McCoy's 5A complete medium (containing 10% fetal bovine serum and 1% P/S) and treated with calcitriol or cisplatin at concentrations of 0-100 μM for 48 h. The treated cells were then mixed with 10% PrestoBlue reagent in culture medium, and incubated for 60 min at room temperature. Afterward, we measured the absorbance at 540 nm with a microplate reader (Molecular Devices, San Jose, CA, USA). All data are expressed as percentages, relative to the control.

Cell proliferation. Cell proliferation was measured by evaluating adenosine triphosphate content, using CellTiter-Glo assay kit (Promega, Madison, WI, USA); the cells were seeded in McCoy's 5A complete medium and treated with calcitriol or cisplatin (0-100 μM) for 48 h. Equal volumes of CellTiter-Glo reagent as cell culture medium were placed in each well. The cells were incubated at room temperature (25˚C) for 10 min, and the luminescence signal was then recorded using a luminescence plate reader (Berthold Technologies, Bad Wildbad, Germany).

Caspase-3/7 activity. Cells were cultured for 24 h in a white-walled 96-well plate and treated with calcitriol or cisplatin (0-100 μM) for 48 h. The treated cells were then incubated with 100 μl of Caspase-Glo 3/7 reagent at room temperature (25˚C) for 30 min. Luminescence was evaluated by a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay by flow cytometry. The apoptotic cell death rate was evaluated by FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA), according to the protocols provided. The cells were seeded in 6-well plates and treated with calcitriol with/without cisplatin (0-100 μM) for 48 h. The supernatant and cells were collected and centrifuged at 1,284 × *g* for 7 min. Cell pellets were resuspended using an FITC Annexin V and PI mixture (comprising 1× binding buffer, FITC Annexin V, and PI), incubated for 15 min at room temperature (25˚C) in the dark. After incubation, 1× binding buffer was added to each sample, and the samples were examined by flow cytometry within 1 h. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (BD Biosciences). Results are presented as percentages of the total number of gated cells.

Silencing RNA (siRNA) and transfection. siRNA for human VDR was synthesized by Genolution (Genolution Pharmaceutical Inc, Seoul, Republic of Korea), and scramble siRNA (sc-37007) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The VDR-targeting siRNA sequence was as follows: 5'-GGAGU UCAUUCUGACAGAU-3'. The transient transfection experiment with siVDR and SCR was performed using Lipofectamine RNAi MAX™ (Invitrogen) according to the manufacturer's instruction. The transfected cells were incubated in humidified chamber with 5% CO_2 at 37˚C for 48-72 h until other assays were performed.

Vascular endothelial growth factor (VEGF) activity. Cells were incubated with or without calcitriol for 48 h at 37˚C. VEGF activity was assessed with VEGF Human ELISA Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Absorbance was measured at 450 nm (Bio-Rad Laboratories, Inc.).

Western blot. SKOV-3 cells were treated with 0-100 μM calcitriol for 48 h. The treated cells were harvested and resuspended in ice-cold cell lysis buffer (Intron Biotechnology, Seongnam, Republic of Korea) containing a Complete Protease Inhibitor Cocktail Mini tablet (Roche Diagnostics, Seoul, Rebuplic of Korea). Protein concentrations were established using a Bicinchoninic acid assay kit (Pierce, IL, USA). Proteins were separated by electrophoresis in 12% or 15% sodium dodecyl sulphate-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and blocked with 5% skim milk. Membranes were incubated with anti-VDR, anti-BCL2 apoptosis regulator (BCL2), anti-VEGF (Abcam, Cambridge, UK), anti-BCL2-associated X (BAX), anti-PARP, anti-cleaved caspase-3 and beta-actin (Cell Signaling Technology, Danvers, MA, USA) antibodies in 5% skim milk or 1% bovine serum albumin. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-secondary IgG antibody (Cell Signaling Technology) being visualized using SuperSignal West Pico Chemiluminescent Substrate (Millipore, Burlington, MA, USA).

Statistical analysis. All data were gained from at least three independent experiments and are presented as mean±SD. Student's *t*-tests were used to demonstrate the significance of differences between different groups. Values of $p<0.05$ were considered statistically significant.

Results

Calcitriol suppressed cell survival and induced apoptotic death of ovarian cancer cells. Calcitriol significantly suppressed cell viability (Figure 1A) and proliferation (Figure 1B) of SKOV-3 cells in a dose-dependent manner. To investigate apoptosis induced by calcitriol in human ovarian cancer cells, we analyzed apoptosis and the apoptosis signaling pathway. Cells treated with calcitriol were assessed using caspase-3/7 activity assays. Caspase-3 activity was significantly increased by calcitriol (Figure

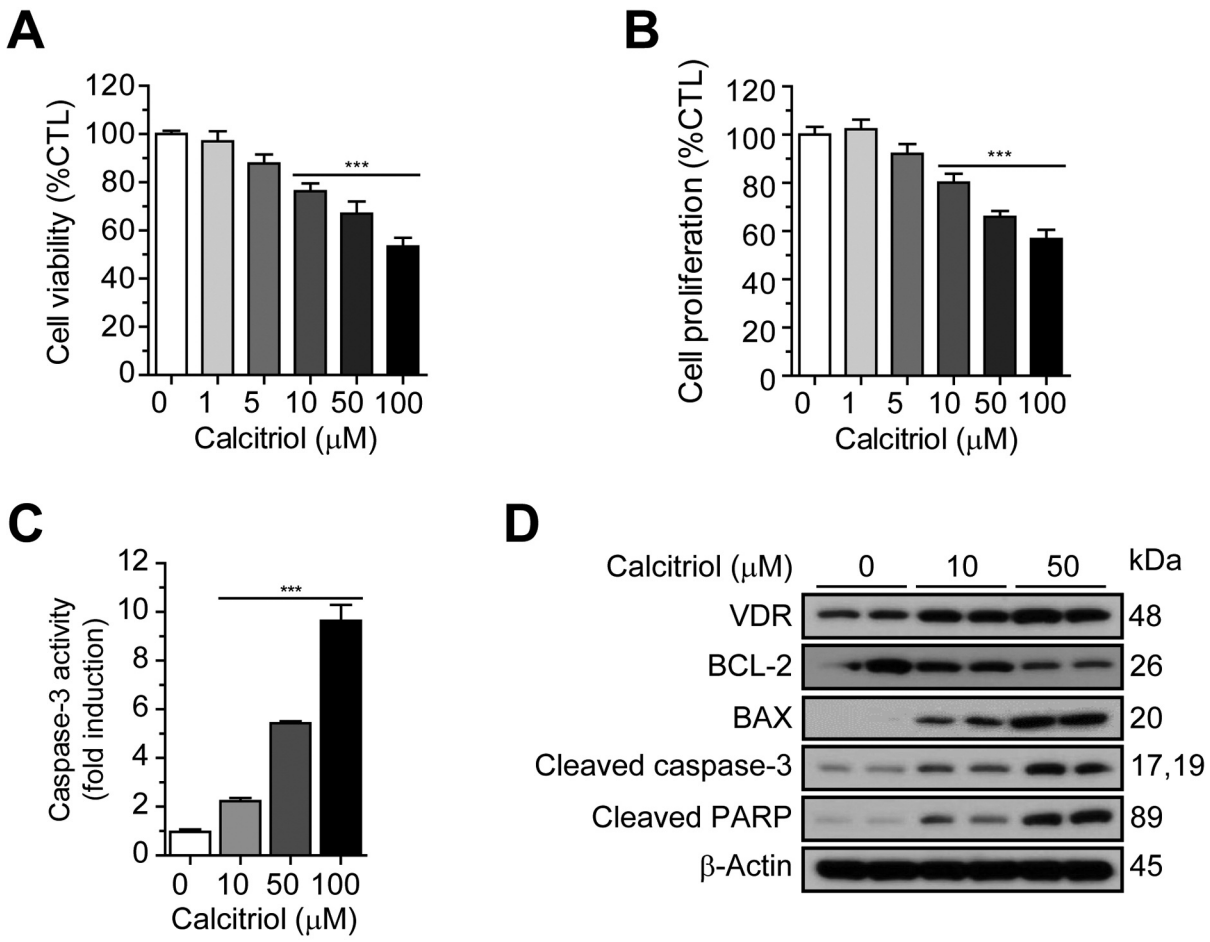

Figure 1. *Inhibition of cell survival and induction of apoptotic cell death by calcitriol in SKOV-3 cells. A: SKOV-3 cells were cultured with 0–100 μM calcitriol for 48 h, and cell viability was determined by PrestoBlue assay. B: The proliferation of calcitriol-treated SKOV-3 cells was determined by measuring adenosine triphosphate content, using CellTiter-Glo assay. C: Caspase-3 activity in calcitriol-treated SKOV-3 cells was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. D: Western blot analysis showing the expression of vitamin D receptor (VDR), BCL2 apoptosis regulator (BCL2), BCL2-associated (BAX), cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) in calcitriol-treated cells. Controls were treated with 0.1% dimethyl sulfoxide. Data are expressed as means±standard deviation. ***Significantly different from the control (CTL) at p<0.001.*

1C). Calcitriol increased the expression of VDR and the proapoptotic proteins BAX, cleaved PARP and cleaved caspase-3, and suppressed the expression of the anti-apoptotic protein BCL2 (Figure 1D). These results suggest that calcitriol inhibits cell survival and induces apoptosis of ovarian cancer cells.

Combined calcitriol and cisplatin therapy inhibited cancer cell survival and promoted apoptotic cell death of ovarian cancer cells. To test the hypothesis that the addition of calcitriol to therapy using platinum-based anticancer drug would reduce cancer cell survival and induce apoptosis of ovarian cancer cells, SKOV-3 cells were co-treated with 0-50 μM calcitriol and 10 μM cisplatin for 48 h. Cells treated with combination therapy exhibited the largest decrease in cell viability (Figure 2A) and proliferation (Figure 2B) when treated with 10 or 100 nM calcitriol with cisplatin compared with single-agent calcitriol or cisplatin. Moreover, when we treated cells with these combinations of calcitriol/cisplatin for 48 h, the caspase-3 activity (Figure 2C) and the percentage of apoptotic cells (Figure 2D) were increased in compared with calcitriol and cisplatin monotherapies. Western blot analysis revealed that the combination therapy of cisplatin and calcitriol in ovarian cancer cells enhanced the expression of pro-apoptotic molecules compared to monotherapies in a dose-dependent manner (Figure 2E). Collectively, these data indicate that combination therapy of calcitriol and cisplatin induced apoptosis of human ovarian cancer cells.

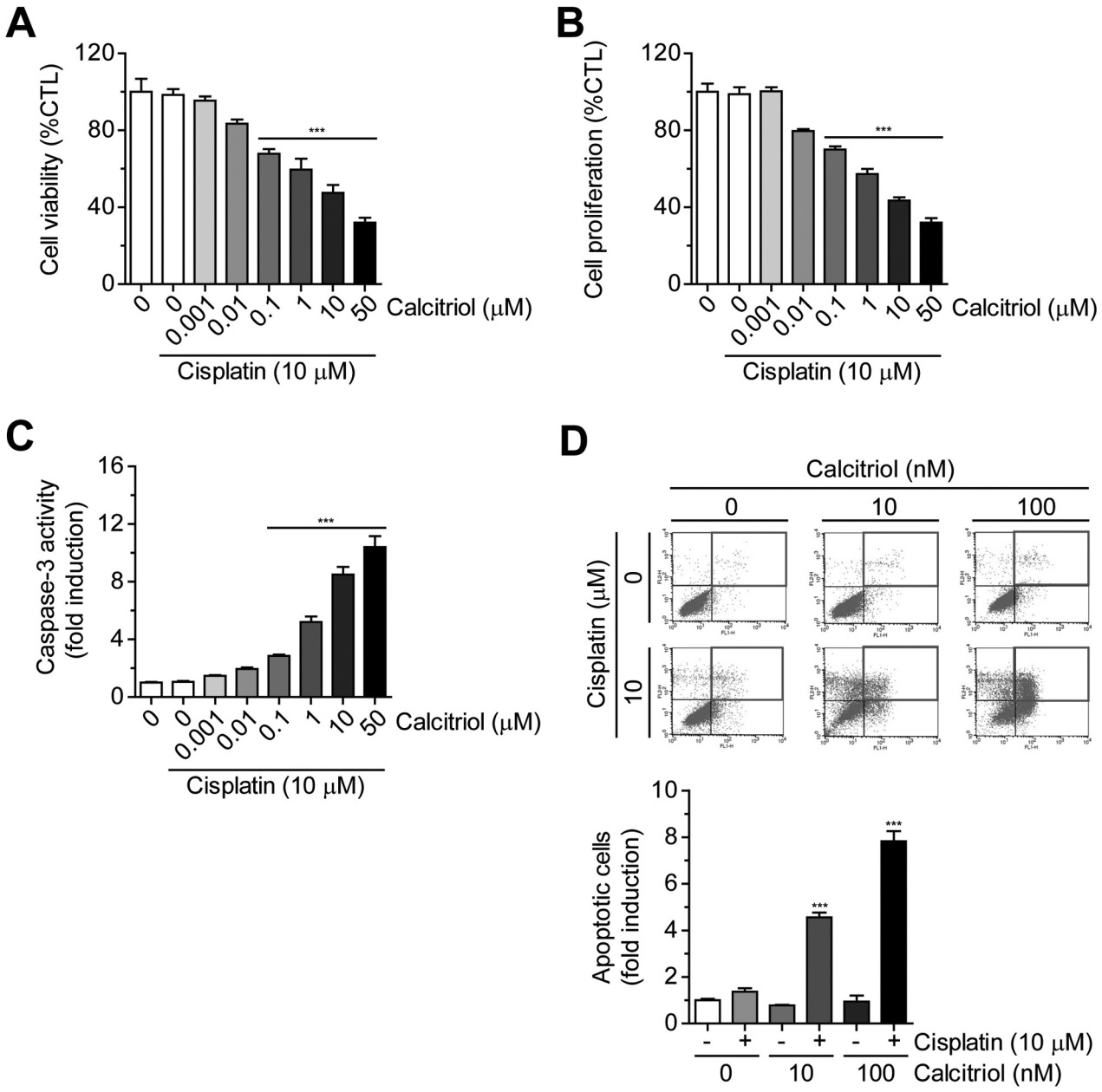

Figure 2. *Effect of combination therapy with calcitriol and cisplatin on cell survival and apoptotic cell death in SKOV-3 cells. SKOV-3 cells were treated with 10 μM cisplatin combined with 0-50 μM calcitriol for 48 h. Cell viability (A) and proliferation (B) were measured using PrestoBlue or CellTiter-Glo assays, respectively. C: Caspase-3 activity in combination-treated SKOV-3 cells was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. D: Flow cytometric analysis using annexin V-fluorescein isothiocyanate/propidium iodide staining of SKOV-3 cells treated with combinations of cisplatin and calcitriol for 48 h (left) and the corresponding quantitative analysis of apoptosis (right). E: Western blot analysis showing the expression of vitamin D receptor (VDR), BCL2 apoptosis regulator (BCL2), BCL2-associated (BAX), cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) in calcitriol-treated cells. Controls were treated with 0.1% dimethyl sulfoxide. Data are expressed as means±standard deviation. ***Significantly different from the control (CTL) at p<0.001.*

Inhibition of cell survival is mediated by regulation of vitamin D activity. To determine whether vitamin D activity contributes to the regulation of cancer cells, we used calcitriol, cisplatin, and their combination to treat siVDR-transfected SKOV-3 cells. VDR knockdown inhibited the effects of the calcitriol–cisplatin combination on cell viability (Figure 3A) and proliferation (Figure 3B). However, VDR knockdown did not alter the effects of cisplatin monotherapy on cell viability or proliferation. Furthermore, we analyzed apoptotic cell death (Figure 3C) and measured caspase-3 activity (Figure 3D) using a luciferase assay and flow cytometry. VDR knockdown significantly suppressed the effect of calcitriol plus cisplatin on the induction of apoptotic cell death and caspase-3 activity. Thus, these findings indicate that the regulation of vitamin D activity mediated the effects of calcitriol plus cisplatin on survival of ovarian cancer cells.

Suppression of VEGF activity by vitamin D. To determine whether vitamin D activity contributes to the regulation of angiogenesis, we performed enzyme-linked immunosorbent assays on SKOV-3 cells treated with calcitriol, cisplatin, and their combination. Calcitriol reduced VEGF activity in SKOV-3 cells (Figure 4A) but this reduction was amplified by its combination with cisplatin (Figure 4B). Thus, our results demonstrate that vitamin D mediates the angiogenesis signaling pathway by regulating VEGF activity in ovarian cancer cells.

Discussion

In this study, the effect of calcitriol and cisplatin combination therapy on the growth of ovarian cancer cells in vitro was verified, to our knowledge, for the first time. This combination therapy inhibited cancer cell growth more than monotherapy did. Furthermore, calcitriol inhibited cell growth and induced apoptotic cell death *via* VDR, and inhibited VEGF.

Several prior epidemiological studies have reported relationships between ovarian cancer and vitamin D (20, 21). The hypothesis that vitamin D is associated with cancer incidence was first proposed in 1980 by the Garland brothers, who reported that people with vitamin D deficiency at higher latitudes have a higher risk of developing malignant tumors (20). Subsequently, their epidemiological studies confirmed that there is a strong inverse correlation between the average daily exposure to UV radiation and ovarian cancer mortality rate (21). In addition, in a case-controlled study of 1,631 female patients diagnosed with EOC, a higher concentration of 25-hydroxyvitamin D was found to be related to longer survival (22). A meta-analysis of 10 cohort studies on the prevalence of ovarian cancer confirmed that an average increase of 20 ng/ml of 25-dihydroxyvitamin D_3 was associated with a relative risk of 0.83 (0.63-1.08) (23).

Experimental studies have reported that calcitriol prohibited the growth of ovarian cancer cells (18, 24, 25), and that it increased apoptosis (17). In the current study, calcitriol alone also led to cell growth inhibition and apoptosis (Figure 1). However, at a physiological concentration, this effect was increased in combination with cisplatin (Figure 2). Moreover, VDR expression increased with calcitriol concentration (Figures 1 and 2). This is consistent with other studies; for example, high levels of 1,25-dihydroxyvitamin D_3 increased VDR expression in ovarian cancer cell lines (17), and other studies have reported that ovarian cancer tissue expressed VDR (24, 26). Therefore, there may be functional vitamin D pathways exploitable for the treatment or prevention of ovarian cancer (27). Furthermore, VDR knockdown reduced the effect of calcitriol (Figure 3), confirming that calcitriol functions *via* VDR. This is consistent with the results of Jiang *et al*., who reported that calcitriol-induced suppression of cancer growth and increased apoptosis were mediated by VDR (18).

Furthermore, VEGF activity was inhibited by calcitriol; this inhibition was increased in combination with cisplatin (Figure 4). Vitamin D has been reported to have a potential role in inhibiting angiogenesis (28, 29). An in vivo study reported that vitamin D reduced the inhibition of tumor-derived epithelial cell growth in VDR-knockout mice, and that the loss of VDR increased the levels of hypoxia-inducible factor-1α (HIF1α), angiopoietin 1, VEGF, and platelet-derived growth factors (28). Increased angiogenesis appears to be involved in cell adaptation to hypoxic conditions, which is regulated by HIF1. HIF1 target genes such as VEGF are regulated *via* HIF-dependent pathways that are inhibited by 1,25-dihydroxyvitamin D3 (29). In the clinic, the cure of advanced and recurrent ovarian cancer involves the use of bevacizumab, a VEGF inhibitor, in combination with platinum-based chemotherapy (30). Therefore, the anti-angiogenesis effect of calcitriol may be helpful in the management of ovarian cancer.

In conclusion, this study suggests a new treatment approach using vitamin D, which is safe and widely consumed on a daily basis. Ovarian cancer is difficult to treat due to high levels of treatment resistance and side-effects associated with anticancer drugs. It may be possible to obtain synergistic effects by adding vitamin D to existing cytotoxic chemotherapy. Moreover, VDR expression might have a role as a predictive biomarker in patients with ovarian cancer.

Conflicts of Interest

None declared.

Authors' Contributions

Ju-Hyun Kim: Conceptualization, methodology, investigation, data curation, original draft, review and editing; Dong Hoon Suh: review and editing; Kidong Kim: review and editing; Jae Hong No: conceptualization, review and editing; and Yong Beom Kim: Conceptualization, methodology, resources and supervision.

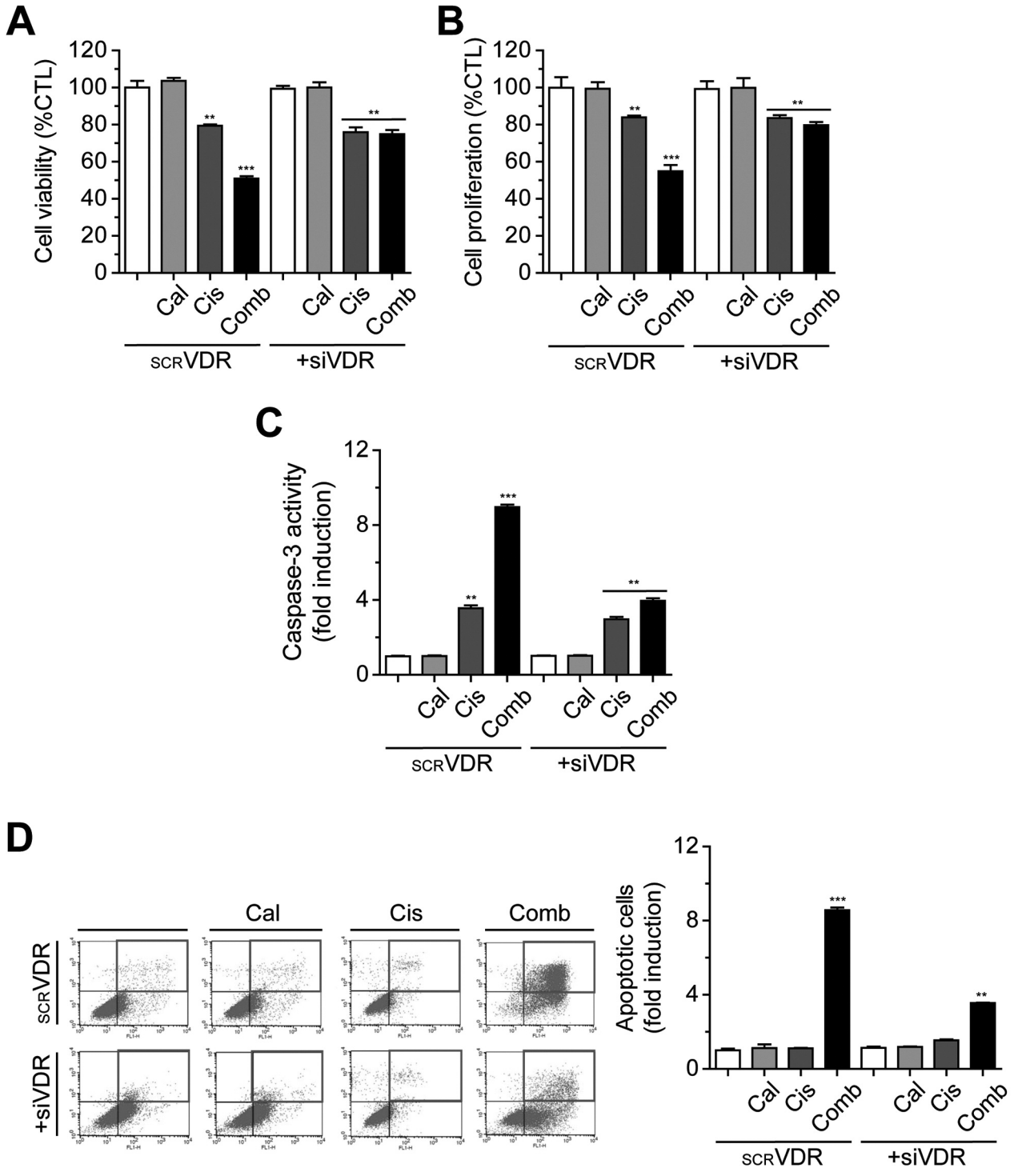

Figure 3. *Effect of the combination (Comb) of calcitriol (Cal) and cisplatin (Cis) therapy on cell survival and apoptotic cell death via regulation of vitamin D receptor (VDR) expression in SKOV-3 cells. SKOV-3 cells were transfected with scrambled (SCR) or silencing (si) RNA for VDR, and treated with 100 nM calcitriol, 10 μM cisplatin, or their combination for 48 h. Cell viability (A) and cell proliferation (B) were measured using PrestoBlue or CellTiter-Glo assays, respectively. C: Caspase-3 activity was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. D: Flow cytometric analysis using annexin V-fluorescein isothiocyanate/propidium iodide staining of SCR or VDR knockdown SKOV-3 cells treated with single-agent or combination therapy. Controls were treated with 0.1% dimethyl sulfoxide. Data are expressed as means±standard deviation. Significantly different from the control (CTL) at: **p<0.01 and ***p<0.001.*

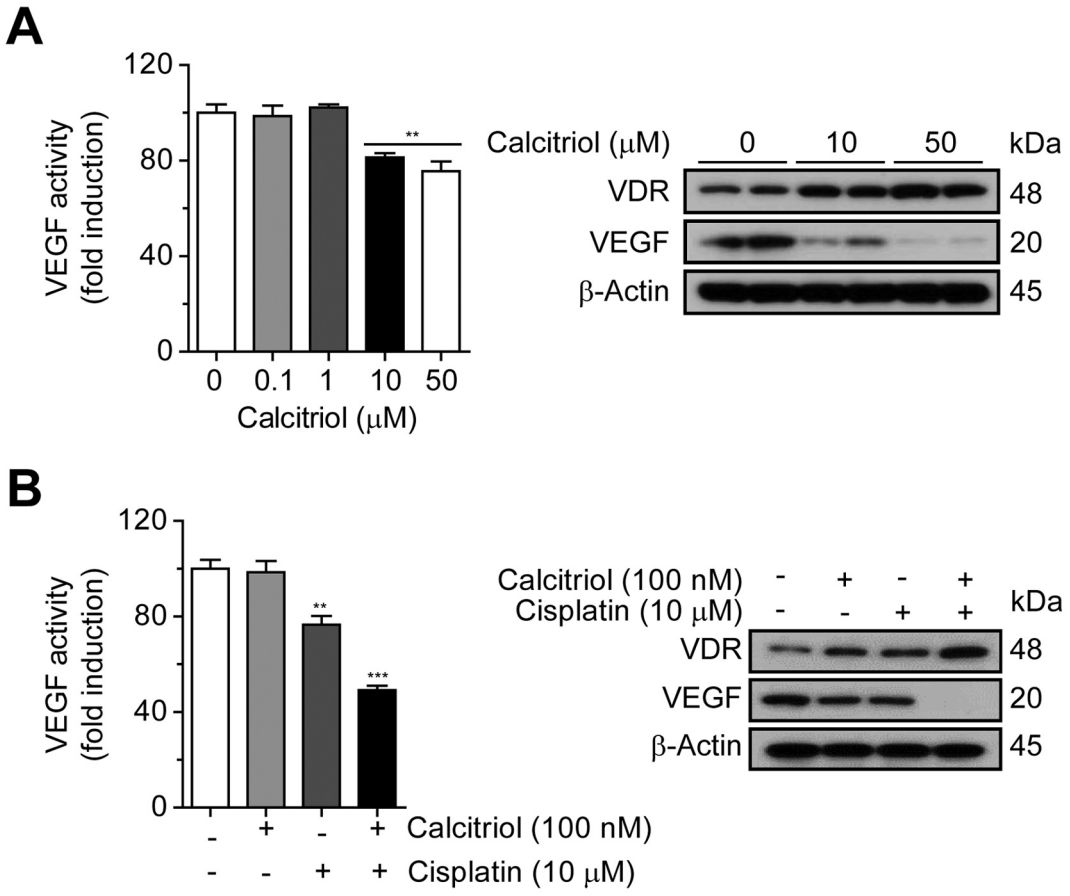

Figure 4. *Inhibition of vascular endothelial growth factor (VEGF) activity by calcitriol and cisplatin combination therapy in SKOV-3 cells. VEGF activity determined via enzyme-linked immunosorbent assay (left) and western blot analysis showing the expression of vitamin D receptor (VDR) and VEGF (right) in cells treated with calcitriol alone (A) and in combination cisplatin (B). Controls were treated with 0.1% dimethyl sulfoxide. Significantly different from the control at: **p<0.01 and ***p<0.001.*

Acknowledgements

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