

## Paclitaxel, Carboplatin and 1,25-D3 Inhibit Proliferation of Endometrial Cancer Cells *In Vitro*

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**Abstract.** *Background/Aim:* Endometrial cancer cells are known to be sensitive to carboplatin and paclitaxel. Furthermore, vitamin D (1,25-D3) has been reported to inhibit endometrial cancer cell growth both as a single agent and combined with carboplatin. However, there are no studies comparing the effect of paclitaxel and carboplatin as single agents vs. in combination in endometrial cancer cell lines. Neither has the effect of 1,25-D3 been studied with paclitaxel. The present study investigated the effect of paclitaxel, carboplatin and 1,25-D3 on the growth of endometrial cancer cells *in vitro*. *Materials and Methods:* Two endometrial adenocarcinoma cell lines (UT-EC-1 and UT-EC-3) were cultured with different doses of paclitaxel, carboplatin and 1,25-D3. The cellular VDR (vitamin D receptor) mRNA levels were measured and the expression of estrogen (ER) and progesterone (PR) receptors by the cells was determined. *Results:* In the UT-EC-1 cell line the growth inhibition was 72% with paclitaxel, 54% with carboplatin and 73% with the

combination of these compounds. The corresponding numbers in UT-EC-3 were 70%, 33% and 65%, respectively. 1,25-D3 suppressed cell growth 88% with paclitaxel, 63% with carboplatin and 87% with their combination in the UT-EC-1 cell line. *Conclusion:* In both cell lines, single-agent paclitaxel was as effective as the combination of the compounds and more effective than single carboplatin. 1,25-D3 may further contribute to the cytotoxic effect of these agents.

Endometrial cancer is the most common gynecological malignancy in developed countries. In Finland incidence is 13.9 per 100,000 persons a year (1). Most women are diagnosed with early-stage disease and have a high cure rate with surgery alone with a reported 5-year survival rate of 90% (2). Still up to 25% of women will have advanced, high grade or recurrent disease and require additional treatment. In these cases, the therapeutic response to cytotoxic and hormonal agents has been typically only partial and of short duration. There is no generally accepted standard chemotherapy in treatment of advanced and recurrent endometrial carcinoma but simultaneous administration of carboplatin and paclitaxel or carboplatin and pegylated liposomal doxorubicin are widely used (3-8).

Vitamin D is an antiproliferative and immunomodulatory secosteroid hormone with a well-established role in maintenance of calcium synthesis. Active metabolite of vitamin D, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25-D3) binds to the vitamin D receptor (VDR) in the cell nucleus. The function and/or expression of the VDR is needed for the

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growth regulatory effects of 1,25-D3 *in vitro* and disabled VDR activity leads to 1,25-D3 insensitivity and accelerated tumorigenesis (9). Many different cell types, including normal and malignant endometrial cells, contain VDR and 1 $\alpha$  hydroxylases to synthesize active 1,25-D3 locally (10, 11). This active metabolite does not enter the circulation and has no effect on calcium homeostasis, but is rather degraded by 24-hydroxylases.

1,25-D3 is well known for its antiproliferative roles including the induction of G<sub>0</sub>-G<sub>1</sub> cell-cycle arrest with associated down-regulation of oncogenic cyclins D1 and D3 and p27/p21 induction (12-15). 1,25-D3 is responsible for VDR-mediated apoptosis and inhibits angiogenesis and differentiation in a variety of cancer types (13, 16-20). 1,25-D3 is a potential antitumor agent because of its ability to regulate cancer cell growth. 1,25-D3 has been found to inhibit endometrial cancer cell growth both as a single agent (21) and combined with carboplatin (22).

Endometrial cancer cells are sensitive to carboplatin, cisplatin and paclitaxel (23, 24). Still there are no studies comparing paclitaxel and carboplatin as single agents *vs.* in combination in endometrial cancer *in vitro* or *in vivo*. In addition, there are no studies examining the effect of vitamin D with paclitaxel or the combination of paclitaxel and carboplatin on endometrial cancer cells. The aim of the present study was to investigate the potential effects of paclitaxel, carboplatin and 1,25-D3 on the growth of endometrial cancer cells both as single agents and in different combinations with each other *in vitro*.

## Materials and Methods

**Cell lines.** Two endometrial adenocarcinoma cell lines were used (UT-EC-1 and UT-EC-3) in this study. The cell lines were originally established from primary tumors before any treatment using an explant technique (25, 26). UT-EC-1 was established from a curettage specimen of a clinical FIGO Stage II Grade 2 endometrial papillary adenocarcinoma and UT-EC-3 from the hysterectomy specimen of a surgical FIGO Stage Ia Grade 3 endometrial adenocarcinoma (23). The biological properties of these cell lines have been described in detail earlier (23).

**Cell cultures and treatments.** Prior to the experiments the cells were cultured as described previously (23). In brief, the cells were plated weekly and maintained in logarithmic phase in 75 cm<sup>2</sup> culture flasks in Dulbecco's modified Eagle minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine and 1% non-essential amino acids at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Both cell lines were tested negative for mycoplasma contamination.

Paclitaxel (Hospira® 6 mg/ml, Maidenhead, UK) and carboplatin (Accord® 10 mg/ml, Middlesex, UK) were provided by the Pharmacy of the Tampere University Hospital. 1,25-D3 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Paclitaxel was diluted in 0.9% sodium chloride to get a 0.1 mM solution. Stock solutions of 100 nM were made in DMEM. Final concentrations

used were 1-100 nM and fresh stock solutions were made for each experiment. Carboplatin was diluted in sterile water to get a solution of 100 µg/ml. The final dilutions used were 0.1-20 µg/ml. The dilutions were made immediately before use. 1,25-D3 was diluted in DMEM to get final 0.1-100 nM solutions. The concentrations of drugs were based on previous reports as well as physiological relevance (23, 24, 27, 28).

**VDR, ER and PR expression analyses.** The VDR mRNA levels in UT-EC-1 and UT-EC-3 cells were measured by qRT-PCR using the LightCycler equipment (Roche, Mannheim, Germany). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was reverse transcribed with SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) as described (29). qRT-PCR was performed using 20 µM gene specific primers (Sigma-Aldrich, sense 5' ATCGCATGATGAAGGAGTT 3', antisense 5' TGCTCCTCAGACAGCTTGG 3') and 10 µM UPL probe (probe number # 12). The PCR program included the following steps: 10 min denaturation at 95°C followed by 45 cycles of 10 s denaturation at 95°C, 30 s annealing at 60°C and 1 s elongation at 72°C. The expression levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPD) housekeeping gene.

The expression of estrogen (ER) and progesterone (PR) receptors in both cell lines was determined with immunohistochemical staining. ER and PR immunostainings were performed using Ventana Benchmark Ultra automated IHC/ISH Slide staining system (Ventana Medical Systems, AZ, USA). Deparaffinization and antigen retrieval were performed using Roche Ventana reagents (EZ Prep 10X concentrate and Cell Conditioning Solution CC1). ER staining was performed using a Roche Ventana RTU Confirm anti-Estrogen Receptor Rabbit monoclonal antibody (Clone SP1) with 32 min incubation time. PR staining was performed using a Roche Ventana RTU Confirm anti-Progesterone Receptor Rabbit monoclonal antibody (Clone 1E2) with 24 min incubation time. Both immunostainings were detected and visualized using Roche Ventana UltraView DAB IHC Detection Kit.

**Cell growth assays.** For each experiment, the cells were plated on 24-well plates at 30,000 cells/well. Cells were then treated with indicated doses of paclitaxel (1-100 nM), carboplatin (0.1-20 µg/ml) and vitamin D<sub>3</sub> (0.1-100 nM) alone or in combination. After a 3-day incubation period, the cells were trypsinized and counted using the Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). All experiments were done in six replicates and were repeated at least twice.

**Statistical analyses.** Non-parametrical Mann-Whitney test was used to assess differences between treated and control groups. Exact *p*-values under 0.05 were considered as statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 23 (IBM SPSS Statistics, IBM Corporation, Chicago, IL).

## Results

The cells were first cultured with different concentrations of paclitaxel (1, 3, 5, 10, 50, and 100 nM) and carboplatin (0.1 µg/ml, 0.5 µg/ml, 5 µg/ml, 10 µg/ml, and 20 µg/ml) to obtain the estimated drug concentration causing at least 50% inhibition of cancer cell growth (IC<sub>50</sub>). In UT-EC-1 cell line

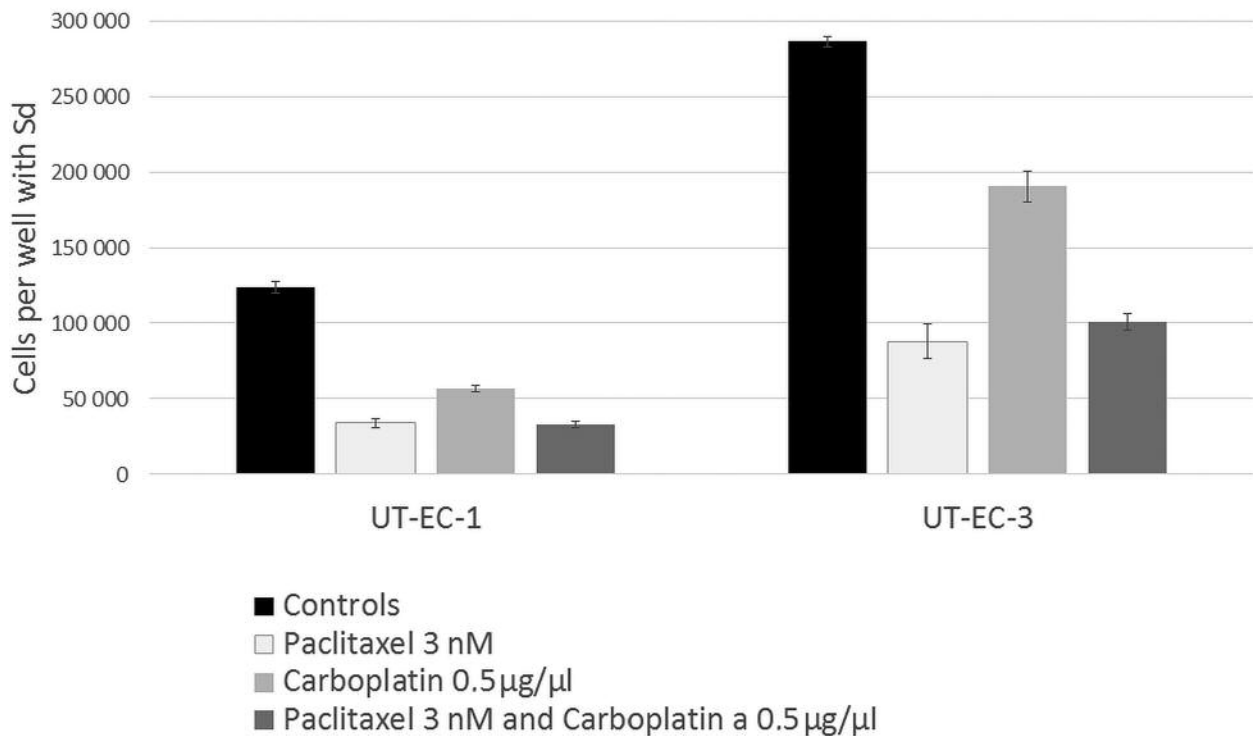


Figure 1. Effect of cytotoxic agents on UT-EC-1 and UT-EC-3 endometrial adenocarcinoma cells growth. The cells were treated for three days with indicated drugs and cell numbers were determined. Mean and SD of six replicates are shown.

the concentration of carboplatin was set in between 0.5-1 µg/ml and the one of paclitaxel was 5 nM. In UT-EC-3 the  $IC_{50}$  was 0.1-1 µg/ml for carboplatin and 1-5 nM for paclitaxel, respectively. A decision was made to use the same concentrations for the both cell lines because the estimated values were so close to each other, 3 nM for paclitaxel and 0.5 µg/ml for carboplatin. Thus, both cell lines were treated either with 3 nM paclitaxel, 0.5 µg/ml carboplatin or with a combination of the two agents and cell growth was compared to untreated controls. In UT-EC-1 cell line growth inhibition with paclitaxel was 72%, with carboplatin 54% and with combination 73%. In UT-EC-3 cell line growth inhibition was 70% with paclitaxel, 33% with carboplatin and 65% with paclitaxel and carboplatin together (Figure 1). In both cell lines, single agent paclitaxel was as effective as the combination of the drugs and more effective than single agent carboplatin. In other words, no additive or synergistic effect was achieved by using them combined with each other. The difference was significant when comparing the effect of single paclitaxel to single carboplatin in both cell lines ( $p=0.002$ ). There was no statistical significance in either one of the cell lines when comparing single paclitaxel to the combination of the paclitaxel and carboplatin ( $p=0.240$ ).

In the second part of the study UT-EC-1 and UT-EC-3 cells were treated with the different concentrations of 1,25-D3 (0.1, 1, 10, 50, and 100 nM) to determine its effect on the growth of the cell lines. There was no growth inhibition with 1,25-D3 in UT-EC-3 cells (Figure 2). In UT-EC-1 cells there was a 24% ( $p=0.002$ ) and 32% ( $p=0.009$ ) growth inhibition with 50 nM and 100 nM doses of 1,25-D3 as compared to the control, respectively (Figure 2). The vitamin D receptor (VDR) expression status was further examined in both cell lines. The UT-EC-1 cells expressed VDR whereas UT-EC-3 did not (Figure 3). Regarding the ER/PR expression, both cell lines were found to be ER- and PR-negative.

The VDR-positive UT-EC-1 cell line was selected for the subsequent experiment. The 50 nM dose of 1,25-D3 was chosen because it is closer to the physiological and clinically attainable concentrations. The cells were cultured with i) 3 nM paclitaxel and 50 nM 1,25-D3, ii) 0.5 µg/ml carboplatin and 50 nM 1,25-D3 and iii) 3 nM paclitaxel, 0.5 µg/ml carboplatin and 50 nM 1,25-D3. Cells were counted as described previously and growth inhibition was analyzed relative to untreated control cells. Paclitaxel and 1,25-D3 suppressed cell growth 88%, carboplatin and 1,25-D3 63% and paclitaxel, carboplatin and 1,25-D3 87% in comparison to the control (Figure 4). Single-agent paclitaxel combined

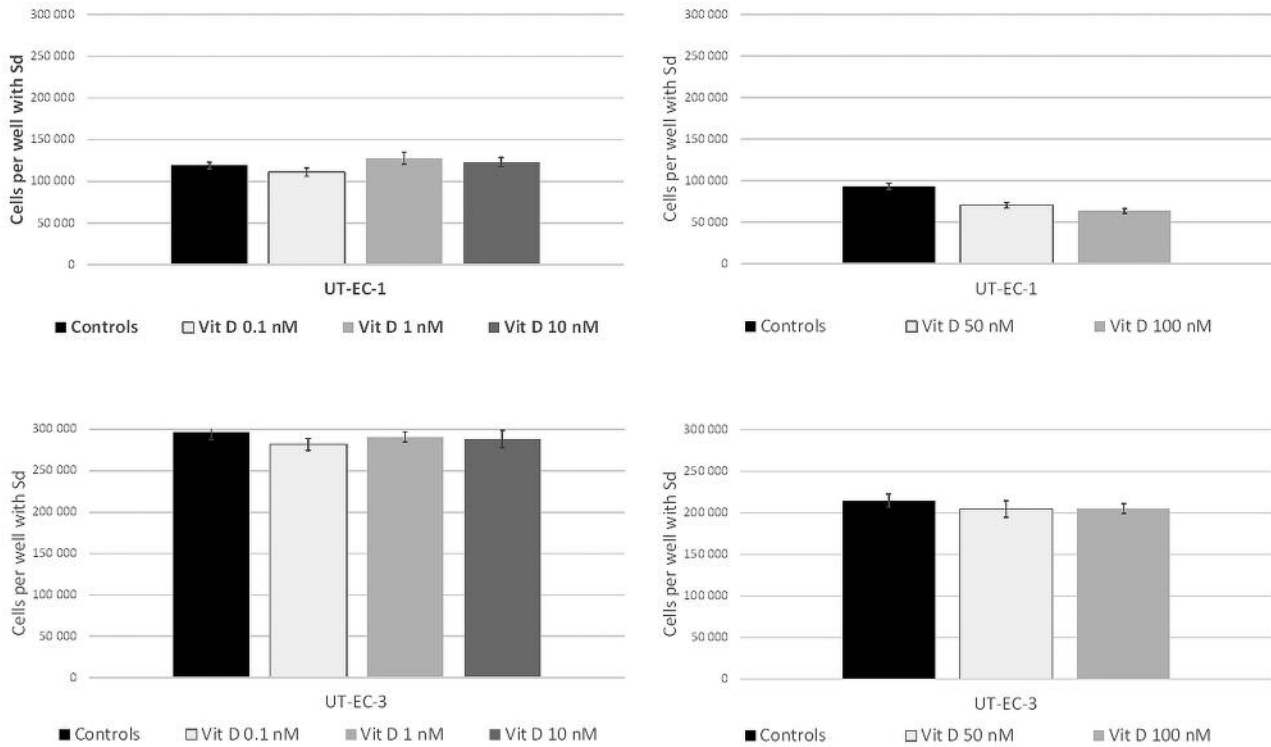


Figure 2. Effect of various concentrations of 1,25-D3 on UT-EC-1 and UT-EC-3 endometrial adenocarcinoma cells growth. The cells were treated for three days with indicated concentrations of 1,25-D3 and cell numbers were determined. Mean and SD of six replicates are shown.

with 1,25-D3 were as effective as the combination of all three ( $p=0.002$ ) and more effective than single agent carboplatin combined with 1,25-D3 ( $p=0.002$ ).

### Discussion

In the present study we tested the effects of paclitaxel and carboplatin as single agents and in combination with and without 1,25-D3 on the growth of endometrial cancer *in vitro*. Our results suggest that paclitaxel alone is as effective as the combination of paclitaxel and carboplatin and more effective than carboplatin alone in inhibiting the growth of endometrial cancer cells. Our data also imply that combining 1,25-D3 with paclitaxel and carboplatin, may further potentiate the growth inhibition of the cancer cells. The additive effect of 1,25-D3 was 17%, 15% and 16% when compared to single-agent paclitaxel, single agent carboplatin and paclitaxel and carboplatin in combination, respectively. On the other hand, adding carboplatin did not provide any additive or synergistic effect, as compared to the combination of paclitaxel and 1,25-D3.

The authors are not aware of any studies comparing clinically single-agent carboplatin and paclitaxel with their combination in endometrial cancer. In phase II studies, the

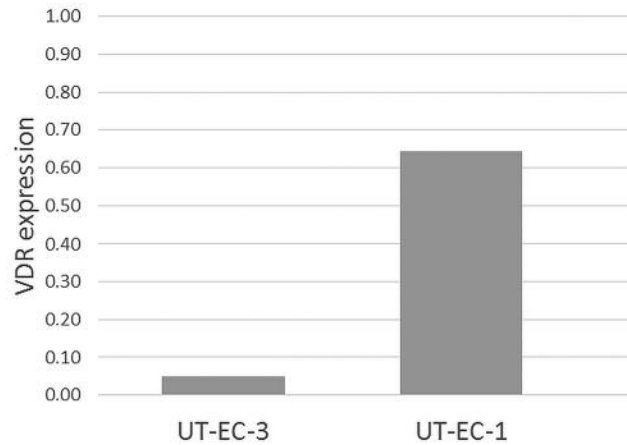


Figure 3. VDR expression in UT-EC-1 and UT-EC-3 endometrial adenocarcinoma cell lines.

response rate of advanced or recurrent chemo-naïve endometrial cancer to both drugs has been in the order of 35% (30, 31). Hoekstra *et al.* (32) compared cytoreductive ability of carboplatin and paclitaxel on endometrial

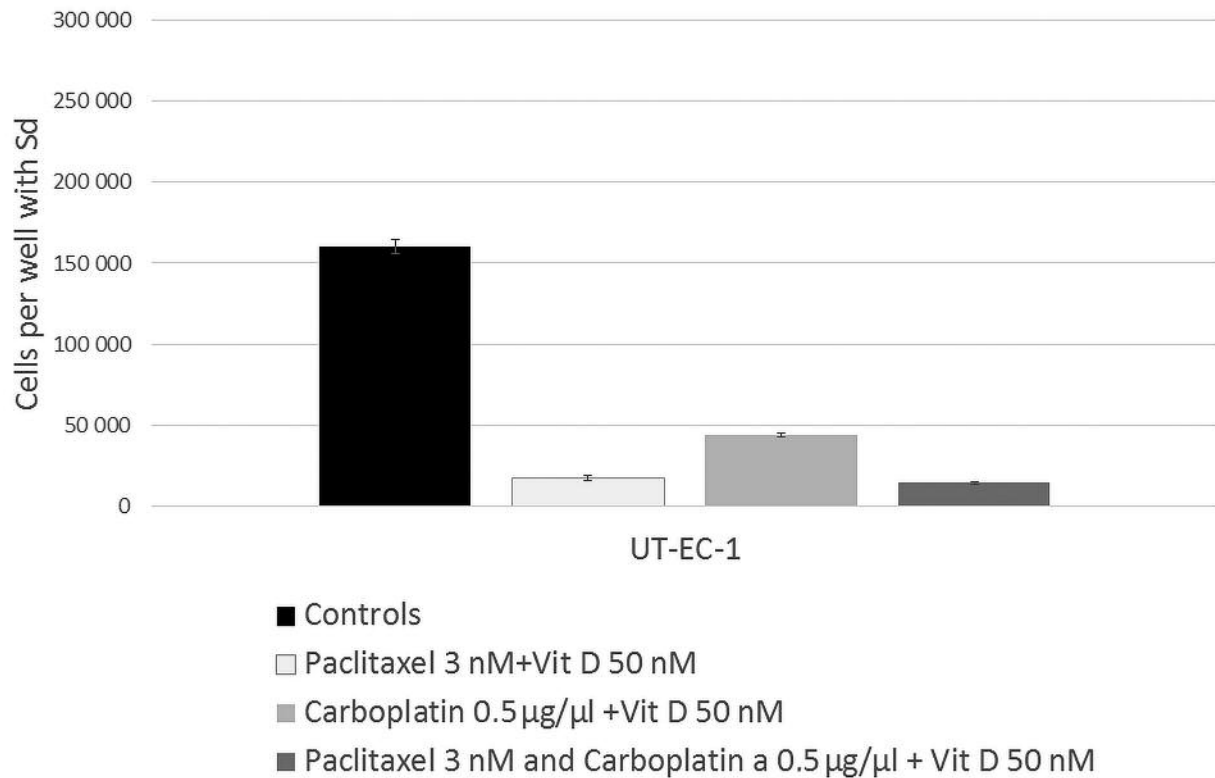


Figure 4. Cell growth inhibition in UT-EC-1 endometrial adenocarcinoma cells with different combinations of cytotoxic agents and 1,25-D3. The cells were treated for three days with indicated drugs and cell numbers were determined. Mean and SD of six replicates are shown.

carcinoma cells. A concentration of 10 nM paclitaxel induced over 90% cell death in 24 h whereas that induced by carboplatin was only 30-40%. This is in line with our results, the cytorreduction rate of paclitaxel was 25% higher in UT-EC-1 cell line and 53% higher in UT-EC-3 cell line than that of carboplatin.

Ballard *et al.* (33) evaluated micro-culture kinetic (MiCK) apoptosis assay to predict response of endometrial cancer cells *in vitro* to single or combination chemotherapy. The results were compared to previous GOG trials. They collected 15 endometrial cancer specimens from total abdominal hysterectomies. All grades, stages and histological types were included. The study demonstrated that 25% of patients might be treated with single-agent chemotherapy. Paclitaxel, ifosfamide and cisplatin had high activity as single agents of which paclitaxel had the highest activity. The correlation of the mean kinetic units with the overall response rates of GOG trials was also high. Single agent paclitaxel appeared to show almost the same activity as the combination of paclitaxel and carboplatin (3.1 vs. 3.4). This is in concordance with our results. The present and previous results imply that it may be reasonable to design at least a

randomized Phase II study comparing paclitaxel alone to the combination of paclitaxel and carboplatin in advanced/recurrent endometrial cancer.

Our preliminary results show that 1,25-D3 may have an additive inhibitory effect with cytotoxic agents in endometrial cancer cells expressing VDR. Paclitaxel and 1,25-D3 had a growth inhibitory effect of 88% compared to 72% of single paclitaxel. A potential bias of the present study is the fact that the experiments were done partly separately. However, we used non-commercial well-established cell lines, and a synergistic anti-tumor effect was seen in UT-EC-1 cell line also with carboplatin and 1,25-D3 (63% vs. 54%), as well as with the combination of all three (87% vs. 73%). Of note is that the 1,25-D3 sensitive UT-EC-1 cell line expressed strongly VDR, while UT-EC-3 did not. Taking the low toxicity of vitamin D into account, it would be interesting to test this phenomenon also clinically.

Earlier studies support the present findings. Saunders *et al.* (22) found additive inhibition of endometrial carcinoma cell growth by carboplatin and calcitriol. Lee *et al.* (17) studied anti-tumor effects of progesterone and 1,25-D3 in endometrial cancer cell lines and immortalized human

endometrial epithelial cells. They found that both agents enhanced vitamin D receptor expression and inhibited cell proliferation through caspase-3 activation. Yabushita *et al.* (21) examined the localization of vitamin D receptor immunohistochemically in 21 endometrial adenocarcinoma specimens, and the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell growth. The VDR was detected in 14 of the 21 specimens. They cultured one cell line expressing VDR with 50 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, resulting in 44% growth inhibition. In contrast, the cells not expressing VDR were completely uninhibited when cultured with 100 nM 1,25-D<sub>3</sub> which is in concordance with our results. According to these findings, endometrial adenocarcinoma is a potential target of 1,25-D<sub>3</sub>.

VDR-negative breast cancer has been found to relapse earlier than VDR-positive one (34). LaPorta *et al.* (35) reported that 1,25-D<sub>3</sub> down-regulates four genes that are known to promote tumorigenesis in breast cancer cells. It has been also demonstrated that the highest expression of VDR, ER and androgen receptors was related to the best prognosis (36). Further, VDR-agonists have been shown to have growth inhibitory effect in established human breast cancer xenografts in animal models (37). 1,25-D<sub>3</sub> has been found to be a potential antiproliferative agent but dose-limiting calcemic effects have been noticed as a major obstacle (37).

Peng *et al.* (38) reported that estrogen receptor-positive breast cancer cells were sensitive whereas estrogen receptor-negative ones were resistant to the antiproliferative effects of vitamin D and its analogs. Furthermore they found that intact VDR-signaling machinery in the cell nucleus is essential for this action. In our study both cell lines were estrogen and progesterone receptor-negative. However, the VDR-positive UT-EC-1 cell line responded to the growth inhibitory effect with 1,25-D<sub>3</sub>. The different VDR status in the cell lines might be due to differences in VDR-signaling machinery and local production and deactivation of the 1,25-D<sub>3</sub>. On the other hand, it seems that in ovarian cancer, expression of VDR is independently regulated from the expression of estrogen and progesterone receptors (39). Whether this also takes place in endometrial cancer cells remains to be elucidated.

In conclusion, we demonstrated with two endometrial carcinoma cell lines that paclitaxel alone seems to be as effective as the combination of carboplatin and paclitaxel *in vitro*. In addition, 1,25-D<sub>3</sub> had a growth-inhibitory effect on the VDR expressing cell line both alone and combined with paclitaxel and carboplatin.

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