Effects of a combination of exemestane and paclitaxel on human tumor cells *in vitro*

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Exemestane, a non-steroidal aromatase inhibitor that shuts down estrogen synthesis, and paclitaxel, an antineoplastic drug, inhibiting microtubule formation and interfering with the cells potential to proliferate, are well established treatments for metastatic breast cancer. Given that exemestane is a treatment for hormone-sensitive tumors in postmenopausal women with more favorable prognosis, while paclitaxel is normally used for women suffering from hormone-insensitive breast cancers with less favorable prognoses, there is currently no experience with the combination of the two drugs. In order to find out to what extent exemestane and paclitaxel add to each other's effects when given concomitantly, the effect of the two drugs alone and in combination on the growth of various gynecological tumor cell lines was assessed. Tumor cell growth was measured according to the cell titer cell proliferation technique, also referred to as the MTS assay, by measurement of relative cell numbers. In gynecological cancer cells expressing aromatase, the effect of a treatment with paclitaxel (10 nM) on cell growth was enhanced by co-treatment with exemestane. This additive effect was independent of ERα expression, but dependent on the presence of androstenedione. It was observed in HEC-1A and Ishikawa endometrial adenocarcinoma cells as well as in SK-OV-3 ovarian cancer and in MDA-MB-231 breast cancer cells. Our findings suggest that a combination of paclitaxel with exemestane might be beneficial for the treatment of aromatase-positive gynecological cancer, because it may allow us to reduce the paclitaxel dosage and therefore the toxicity of the treatment. *Anti-Cancer Drugs* 15:55–61 © 2004 Lippincott Williams & Wilkins.

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Introduction

CYP450^{arom}, also referred to as aromatase, is a key molecule in certain types of breast cancer. Since aromatase catalyzes the synthesis of estradiol and estrone, it provides a perfect therapeutic target for the inhibition of estrogen synthesis both in the body and in the tumor [1,2].

Aromatase inhibitors are divided into two classes, type I and type II. The difference between the two classes relates both to their mode of action and their chemical nature. Type II aromatase inhibitors, like anastrozole and letrozole, bind reversibly to the aromatase and have non-steroidal structures. Type I aromatase inhibitors, also known as aromatase inactivators, bind irreversibly to aromatase and are derived from androgens. The most widely used aromatase inactivator is exemestane [3].

This drug effectively suppresses the aromatase activity both in the body system and tumor cells, thus lowering the plasma estrogen levels by at least 95%. Exemestane has been evaluated extensively in various clinical trials, including a large phase III study, where exemestane was compared to megestrol acetate [4]. Exemestane not only prolongs the survival time, but also offers a well-tolerated treatment option for postmenopausal women with hormone-sensitive metastatic breast cancer [5,6].

We have investigated whether a cytotoxic drug like paclitaxel could be combined with exemestane to a therapeutic approach in a way that could allow a dose reduction of the cytotoxic component. We set up a study to test the effects exemestane and paclitaxel have on the growth of different gynecological cancer cell lines when given concomitantly, compared to each drug alone.

Materials and methods

Cell culture

BG-1 and SK-OV-3 ovarian cancer cell lines, MCF-7 and MDA-MB-231 breast cancer cell lines, and HEC-1A and Ishikawa endometrial cancer cell lines (obtained from ATCC, Manassas, VA) were maintained in phenol red-free DMEM supplemented with 10% fetal calf serum (FCS), 100 μg/ml penicillin, 100 μg/ml streptomycin and

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10^{-5} M androstenedione (Life Technologies, Grand Island, NY). All cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Drug treatment and cell growth analysis

FCS was purchased from Gibco/BRL, phenol red-free DMEM medium (with 1000 mg/l glucose and with L-glutamine) and Serum Replacement 2 (SR2) were obtained from Sigma (Germany). Exemestane [7,8] was kindly provided by Pharmacia Oncology (Germany) and paclitaxel was purchased from Sigma. The drug stock solutions (10^{-5} M) were prepared by dissolving exemestane in DMF and paclitaxel in DMSO, and stored at 4°C. All drugs were diluted to working concentration in culture medium.

Cells were suspended in phenol red-free DMEM containing 5% FCS medium and seeded in 96-well plates (Nunc, Denmark) (3×10^3 cells/well). For the reduction of serum concentration, the medium was changed (0.5% FCS) after 24 h and, after 48 h, the cells were washed in PBS and incubated in SR2 medium (Sigma) ($\pm 10^{-5}$ M androstenedione) containing the test substances in quadruplicates. The final concentration of DMF and DMSO in the culture medium was 0.1%. Controls were exposed to culture medium containing the same quantity of DMF and DMSO without drugs (vehicle). Cell viability was determined using the Trypan blue exclusion method (usually more than 95%). All experiments were performed in quadruplicates and repeated 3 or 4 times.

After 24 or 96 h of treatment, cell growth was evaluated using an MTS assay according to the manufacturer's instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). The assay utilizes the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). Briefly, cells were seeded in 96-well plates (four wells for each group, 3×10^3 cells/well in 0.2 ml). At the end of the incubation period, 20 µl of MTS reagent was added to each well and 1–4 h later, plates were read at 490 nm using a Multiscan microplate reader (Germany).

RNA isolation

RNA was isolated from cultured cells using a procedure described in Unit 4.1 of *Current Protocols in Molecular Biology*. Briefly, cells were washed with ice-cold phosphate-buffered saline. The pellets of harvested cells were resuspended in a lysis buffer (10 mM Tris–HCl, pH 7.8, 1.5 mM $MgCl_2$, 150 mM NaCl and 65% NP-40) and incubated for 5 min on ice. The intact nuclei were removed by a brief microcentrifuge spin and an equal volume of a urea-containing buffer (100 mM Tris–HCl, pH 7.4, 7 M urea, 0.35 M NaCl, 1% SDS and 10 mM EDTA) was added to the cytoplasmic supernatant to denature proteins. Proteins were removed by extractions with phenol/chloroform. The cytoplasmic RNAs were recovered by ethanol precipitation, and quantitated by measuring their absorbance at 260 and 280 nm.

RT-PCR analysis

We designed intron-spanning oligonucleotide primers binding in exon 5/6 and exon 8 for the examination of the human aromatase gene expression in all cell lines. β-Actin was used as a RT-PCR systemic control. The aromatase primer set was forward 5′-GAGGATCCCTTTGGACGAA-3′ and reverse 5′-TGCGATCAGCATTTCCAAT-3′. The β-actin primer set was forward 5′-CTGTGGCATCCACGAAACTA-3′ and reverse 5′-CGCTCAGGAGCAATG-3′. RNA isolated from cultured cells was subjected to RT-PCR (SuperScript One-Step RT-PCR with Platinum Taq; Life Technologies) according to a procedure described by the manufacturer. Specifically, RT-PCR reactions were carried out in a total volume of 50 µl containing 1 µg of total RNA, 20 pmol of each primer and 1 µl reverse transcriptase/Platinum Taq Mix. The reverse transcription reaction was carried out at 50°C for 30 min and pre-denatured at 94°C for 2 min. PCR was performed for 30 cycles using the following temperature profile: 94°C, 1 min (denaturation), 55°C, 1 min (primer annealing) and 72°C, 2 min (primer extension). A final extension cycle was performed for 10 min at 72°C before cooling the reaction mixture to 4°C. RT-PCR products were analyzed using 1.5% agarose gel electrophoresis.

Data analysis

Data obtained from three experiments were pooled and expressed as percentage of control cells (100%). Statistically significant differences between treatment were determined by one-factor analysis of variance (ANOVA) followed by Dunnett's post-test; p values less than 0.05 were considered as statistically significant.

Results

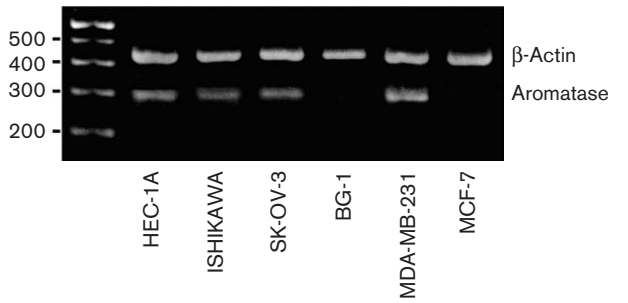
Aromatase and estrogen receptor (ER) α expression

In order to examine aromatase expression in the gynecological cancer cell lines employed in our experiments, we performed a RT-PCR analysis. Expression of aromatase was detected in SK-OV-3, MDA-MB-231, HEC-1A and Ishikawa cells, but not in MCF-7 and BG-1 cells (Fig. 1). ERα expression could be demonstrated by means of additional RT-PCR experiments in BG-1, MCF-7 and Ishikawa cells, but not in MDA-MB-231, SK-OV-3 and HEC-1A cells (data not shown) [9–12]. Therefore, these experiments revealed different combinations of aromatase and ERα expression in our cell culture models (Table 1).

Effect of exemestane and paclitaxel on the growth of gynecological cancer cell lines

In a first set of proliferation assays, we investigated the effects of exemestane and paclitaxel as single substances

Fig. 1



RT-PCR analysis of aromatase expression in gynecological cancer cell lines. The 307-bp aromatase amplificate was detected in HEC-1A, Ishikawa, Sk-OV-3 and MDA-MB-231 cells. Detection of 454-bp β-actin mRNA expression was used as a positive control.

Table 1 Expression of aromatase and ERα in gynecological cancer cell lines

	Aromatase	ERα
BG-1	–	+
SK-OV-3	+	–
MCF-7	–	+
MDA-MB-231	+	–
HEC-1A	+	–
Ishikawa	+	+

on the growth of cancer cell lines derived from human breast, ovary and endometrium. The breast cancer cell lines MCF-7 and MDA-MB-231, the ovarian cancer cell lines BG-1 and SK-OV-3, and the endometrial adenocarcinoma cell lines HEC-1A and Ishikawa were cultured in DMEM supplemented with 10% FCS and treated with 1–100 nM exemestane or paclitaxel for 96 h. When treated with exemestane, only aromatase-positive cell lines showed an antiproliferative effect of this substance at the highest concentration (100 nM). Additionally, the effects of exemestane were analyzed in serum-free culture (Fig. 2). Under serum- and estradiol-free culture conditions (SR2) without androstenedione supplementation, no significant antiproliferative effect of 100 nM exemestane could be observed. In contrast, when cultured in serum-free medium containing exemestane and 10 μM androstenedione, the proliferation of the aromatase-positive cell lines was significantly decreased after 96 h. In the aromatase-negative cell lines MCF-7 and BG-1, no effect of exemestane was observed irrespective the presence of androstenedione.

In order to determine a concentration of paclitaxel in our cell culture models that does not cause complete reduction of cell growth and thus could be used for the combination treatment, cell lines were treated with different concentrations of paclitaxel for 96 h. We observed a dose-dependent effect of paclitaxel, with a maximal decrease of measured cell numbers after treatment with 100 nM of this substance. Treatment with 10 nM paclitaxel only resulted in a slight reduction of cell numbers after 96 h (Fig. 3).

Combination treatment with exemestane and paclitaxel

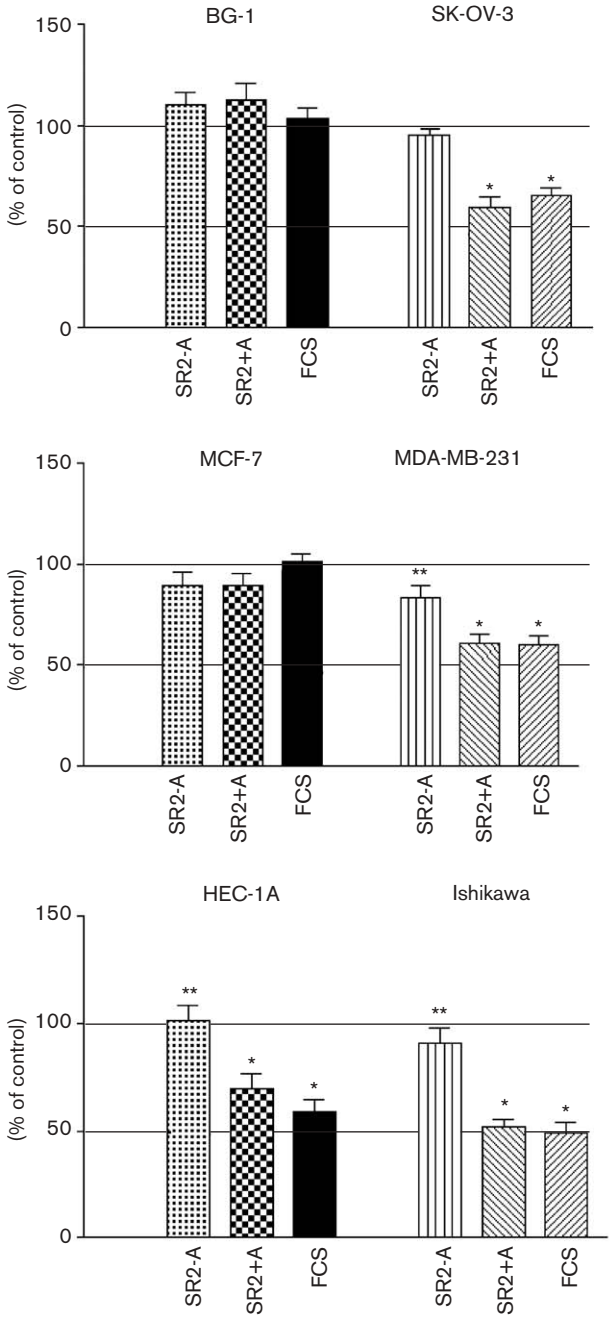
In order to examine the effect of a combination treatment on the growth of gynecological tumor cells, these cells were treated with 100 nM exemestane in combination with low-concentration paclitaxel (10 nM). When cultured in medium containing 10% FCS or serum- and estradiol-free medium with androstenedione supplementation, the combination of exemestane and paclitaxel led to a significantly larger inhibition when compared to either exemestane or paclitaxel treatment alone. This effect was observed in aromatase-positive cell lines only. We measured an additive antiproliferative effect of both substances after treatment of SKOV-3, MDA-MB-231, HEC-1A and Ishikawa cells cultured in 10% FCS or serum-free medium supplemented with androstenedione. The maximal inhibitory effect on cell proliferation was observed when the aromatase-positive cell lines were cultured in serum-free medium supplemented with androstenedione after treatment with 100 nM exemestane and 10 nM paclitaxel for 96 h (Fig. 4). However, in serum- and estradiol-free medium not containing the aromatase substrate androstenedione, the combination of exemestane and paclitaxel did not exhibit a significant additional effect compared to either exemestane and paclitaxel alone.

In the aromatase-negative cell lines in MCF-7 and BG-1, the combination of exemestane and paclitaxel resulted in no benefit regarding the inhibition of cell proliferation irrespective of the medium type.

Discussion

Exemestane is mainly used for the therapy of breast cancer patients expressing estrogen receptors [13,14], because the drug affects the supply of estrogens to the tumor, resulting in a significant decrease in serum estrogen concentration, primarily by inhibition of estrogen production [15]. Effects of exemestane on breast cancer cell lines have been thoroughly examined, but relatively little is known about its action in endometrial and ovarian tumor cells, although aromatase has been demonstrated to be expressed in endometrial adenocarcinoma and ovarian cancer [16,17]. We used breast, endometrial and ovarian cancer cell lines that provide a model for our studies of the exemestane effect on these kinds of carcinoma. Our results demonstrated inhibitory effects of 100 nM exemestane on the growth of aromatase-positive cell lines in serum-supplemented medium. To confirm the specificity of the exemestane effect, we compared the inhibitory effects of this substance on cell lines cultured in defined, serum-free medium with or without the presence of the aromatase substrate androstenedione. Antiproliferative effects of exemestane were observed in aromatase-positive cells cultured in the presence of androstenedione only, confirming the specificity of the exemestane action. The absence of aromatase expression or androstenedione supplementa-

Fig. 2

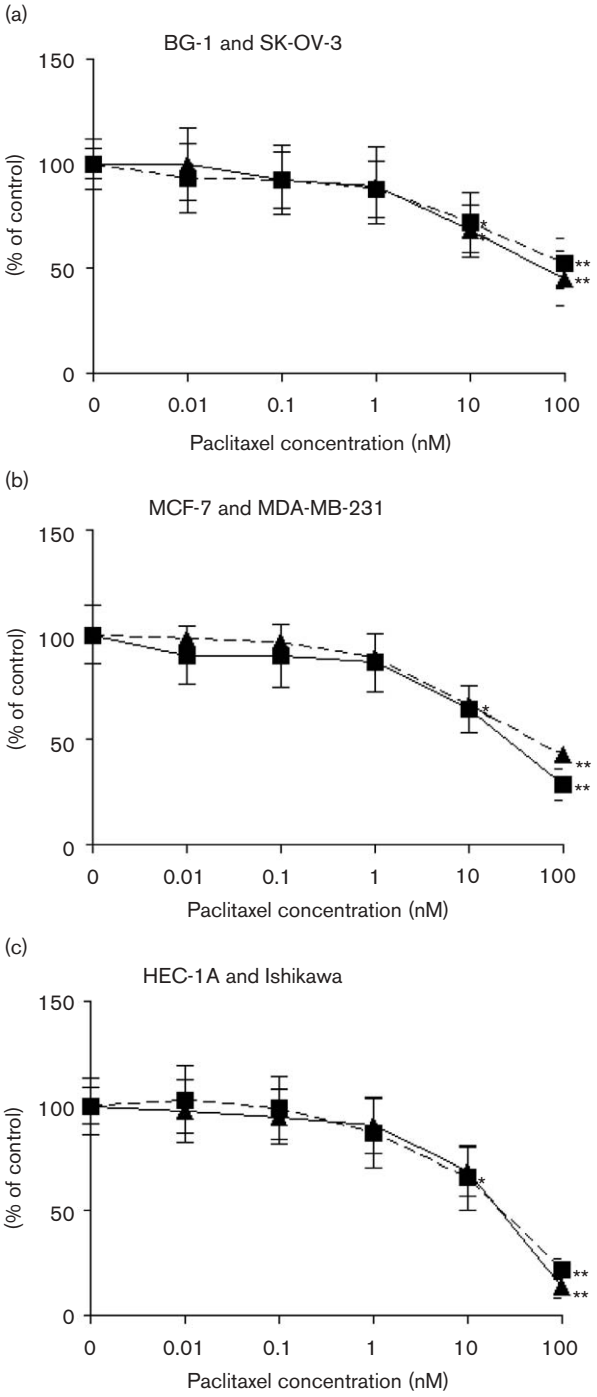


Effects of exemestane on the growth of gynecological cancer cells. Cells were cultured in 10% FCS (FCS) or serum-free medium (SR2) with or without androstenedione (SR2 + A, SR2-A) and treated with 100 nM exemestane. After 96 h, cell growth was quantified using the cell titer cell proliferation (MTS) assay, expressed as percentage of the exemestane-free medium control (here defined as 100%). Results are shown as mean ± SEM of three independent experiments in quadruplicate. *$p < 0.05$ SR2 + A or FCS versus SR2-A or control.

tion was sufficient for the lack of any exemestane effect on cellular proliferation. The absence of aromatase expression in BG-1 was consistent with previous data classifying this cell line as aromatase-negative [18]. Our data did not support a previous study reporting the expression of aromatase in low levels in MCF-7 cells [19], since the aromatase RT-PCR produced no specific fragment and no exemestane effects could be observed even in the presence of androstenedione.

Our results supported a previous report that MDA-MB-231, HEC-1A and SK-OV-3 were ER-negative [9–11]. However, the androstenedione-dependent antiproliferative effect of exemestane suggests the presence of an autocrine mechan-

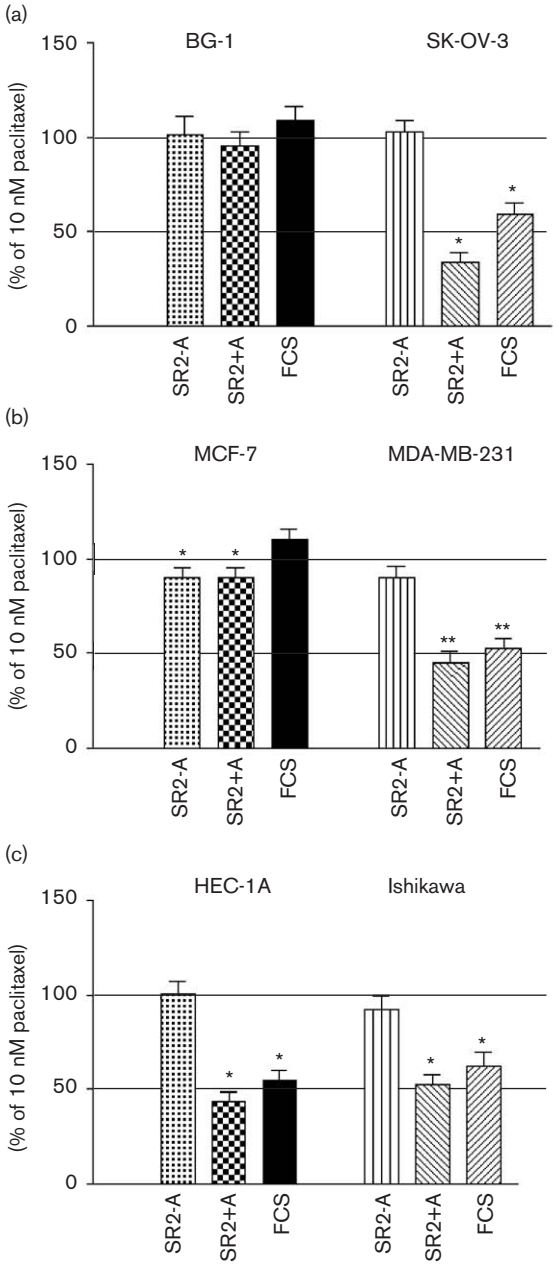
Fig. 3



Effects of paclitaxel on the growth of gynecological cancer cells. Cells were cultured in 10% FCS medium and treated with 0.01–100 nM paclitaxel. After 96 h, cell growth was quantified using the cell titer cell proliferation (MTS) assay. The data from four independent experiments run in quadruplicate are shown as mean ± SEM, expressed as percentage of the paclitaxel-free medium control. *$p < 0.05$ versus control, **$p < 0.01$ versus control. (a) BG-1 (squares), SK-OV-3 (triangles), (b) MCF-7 (squares), MDA-MB-231 (triangles) and (c) HEC-1A (squares), Ishikawa (triangles).

ism for the stimulation of proliferation by estradiol in these cell lines. There have been a number of reports showing that estrogen can have effects that cannot be accounted for by the classical model of nuclear ER activating gene transcription [20]. It has been demonstrated in previous studies that cellular response to estradiol stimuli is mediated both by classical ER binding to estrogen response elements and by activation of mitogen-activated protein

Fig. 4



Effects of a combination treatment with exemestane (100 nM) and paclitaxel (10 nM) on cell growth. Additive exemestane effects are shown in relation to the measured cell growth after treatment with 10 nM paclitaxel alone (here defined as 100%). Cells were cultured in 10% FCS (FCS) or serum-free medium (SR2) with or without androstenedione (SR2 + A, SR2-A) and treated with 100 nM exemestane, 10 nM paclitaxel or combinations of both substances. After 96 h, cell growth was quantified using the cell titer cell proliferation (MTS) assay, expressed as percentage of the 10 nM paclitaxel medium control. Results were shown as mean ± SEM of three independent experiments in quadruplicate. (a) $*p<0.01$ versus control or SRA-2. (b) $**p<0.01$ versus SRA-2 or control. (c) $*p<0.05$ versus SRA-2 or control.

kinase signal transduction [21,22]. The data suggest the involvement of ERα-independent mitogenic signaling cascades in the estradiol-induced proliferative response of our aromatase-positive cancer cell lines.

In our cell culture models, paclitaxel treatment led to a dose-dependent decrease of measurable cell numbers, with a maximal decrease at a concentration of 100 nM.

In this study, we demonstrated that a combination of exemestane with a relatively low concentration of paclitaxel (10 nM) effectively reduced the growth of gynecological tumor cells in a diversity of aromatase-positive cell lines and that androstenedione was necessary for the action of exemestane. The effect of the combination was additive and superior to single-agent

therapy. Exemestane given alone exhibited a growth-suppressive activity in MDA-MB-231, HEC-1A, Ishikawa and SK-OV-3 cells only in the presence of androstenedione which is converted to estrone and estradiol by the catalysis of aromatase. Androstenedione was not only the substrate of aromatase activity, but also the key intermedium where exemestane inhibits cellular proliferation. Even treatment with 100 nM of exemestane by itself did not eliminate tumor cell growth completely. However, the combination with a low dose of paclitaxel resulted in growth suppression comparable with the effect of a treatment with a high dose (100 nM) of paclitaxel alone.

Although the potential clinical relevance of our *in vitro* findings is currently difficult to judge, our data suggest that the dosage of paclitaxel might be reduced when combined with exemestane, leading to a minimized toxicity of the treatment. However, the inhibitory effects of exemestane were statistically significant at 100 nM only when compared to the control. Therefore, it would be necessary to find an appropriate dosage *in vivo* when combined with paclitaxel. Because exemestane is generally well tolerated if compared to paclitaxel, it will have to be examined in future *in vivo* studies whether the addition of exemestane is able to produce an additive and concentration-dependent effect which cannot only effectively enhance the antitumoral effects of paclitaxel, but can also reduce the toxicity by lessening the dosage of paclitaxel.

In conclusion, our findings suggest that a combination of paclitaxel with exemestane might be beneficial for the treatment of aromatase-positive gynecological cancer, because it may allow us to reduce the paclitaxel dosage and therefore the toxicity of the treatment.

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