ERα-Targeted Therapy in Ovarian Cancer Cells by a Novel Estradiol-Platinum(II) Hybrid

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As we previously showed, we have synthesized a new family of 17β-estradiol-platinum(II) hybrids. Earlier studies revealed the VP-128 hybrid to show high efficiency compared with cisplatin toward hormone-dependent breast cancer cells. In the present research, we have studied the antitumor activity of VP-128 in vitro and in vivo against ovarian cancer. In nude mice with ovarian xenografts, VP-128 displayed selective activity toward hormone-dependent tumors and showed higher efficiency than cisplatin to inhibit tumor growth. Similarly, in vitro, transient transfection of estrogen receptor (ER)-α in ERα-negative A2780 cells increased their sensitivity to VP-128-induced apoptosis, confirming the selectivity of VP-128 toward hormone-dependent tumor cells. In agreement, Western blot analysis revealed that VP-128 induced higher caspase-9, caspase-3, and poly (ADP-ribose) polymerase cleavage compared with cisplatin. The activation of caspase-independent apoptosis was also observed in ERα-negative A2780 cells, in which VP-128 rapidly induced the translocation of apoptosis-inducing factor to the nucleus. Conversely, subcellular localization of apoptosis-inducing factor was not modified in ERα-positive Ovcar-3 cells. We also discovered that VP-128 induces autophagy in ovarian cancer cells because of the formation of acidic vesicular organelles (AVOs) and increase of Light Chain 3B-II protein responsible for the formation of autophagosomes; pathways related to autophagy (AKT and mammalian target of rapamycin) were also down-regulated, supporting this mechanism. Finally, the inhibition of autophagy using chloroquine increased VP-128 efficiency, indicating a possible combination therapy. Altogether these results highlight the beneficial value of VP-128 for the treatment of hormone-dependent ovarian cancers and provide preliminary proof of concept for the efficient targeting of ERα by 17β-estradiol-Pt(II)-linked chemotherapeutic hybrids in these tumors. (Endocrinology 154: 2281–2295, 2013)

In North America, ovarian cancer accounts for one fifth of gynecological cancer and is the fifth leading cause of cancer death among women (1–4). Among gynecological cancers, the majority overexpress the estrogen receptors (ER; mainly ERα) as compared with normal tissues (4–7) and many can be classified as hormone dependent. Estrogen binds to its receptor, ERα/β, which then translocates from the cytoplasm to the nucleus to act as a transcription factor (8–10). Estrogen in ovarian cancer has high probabilities to promote cellular proliferation, malignancy, and neoplastic transformation (7, 11). Thus, using ERs as a biological target may provide a useful tool for the development of endocrine therapies and has already been validated with drugs such as tamoxifen (3, 12).

During the last decades, the most active chemotherapeutic drugs used in ovarian cancer have been platinum drugs, cisplatin, and carboplatin (13). Cisplatin [cis-diamminedichloroplatinum(II)] acts on fast-growing cells, a common characteristic of cancer cells, by intercalating into DNA, stopping cell division, and then ultimately leading to cell death (14). It is well known that platinum drugs are potent apoptosis inducers (15). Apoptosis is charac...
terized by morphological changes such as cytoplasmic condensation and fragmentation of DNA at specific sites forming apoptotic bodies (16). However, it has been recently shown that cisplatin can also trigger autophagy (17). Autophagy is characterized by the presence of autophagic vacuoles in cytoplasm and the enlargement of Golgi and endoplasmic reticulum. Autophagy normally occurs during nutrient starvation for recycling but can also occur without needing starvation upon treatment of platinum compounds (16). Its mechanism consists of formation of membrane of the autophagosome around damaged organelles and proteins after fusion with lysosome, leading to the degradation of the items for recycling (18). In cancer cells, autophagy induced by chemotherapeutic drugs is often considered as chemoprotective, suggesting it could be implicated in chemoresistance. In agreement, inhibition of autophagy in several of these cancer cells improves efficiency of cisplatin for the induction of apoptosis (18–21).

The presence of severe side effects such as nephrotoxicity and hematotoxicity is the main drawback of platinum analogues due to the absence of selectivity toward cancer cells (22–25). Because the estrogen receptor (ERα) is highly expressed in hormone-dependent ovarian cancer, 17β-estradiol (E2) used as a carrier molecule of cisplatin could increase selectivity and efficiency of the drug. We have recently reported the synthesis of a new family of E2-platinum(II) hybrids (26, 27). Previous studies revealed that one of these hybrids, VP-128, showed high efficiency compared with cisplatin toward breast cancer cells and was also selective toward ERα+ breast cancer xenografts in nude mice (28). In the present study, we have investigated for the first time, in vitro and in vivo, the antitumor activity of VP-128 using human ERα-positive (Ovar-3, Skov-3) and ERα-negative (A2780 and A2780CP-cisplatin resistant variant) ovarian cancer cells. We have also investigated the mode of action of VP-128 in ovarian cancer cells to induce apoptosis and autophagy.

**Materials and Methods**

**Cell culture**

Human ovarian cancer cell lines Skov-3 (HTB-77) (ERα positive) and NIH:Ovar-3 (HTB-161) (ERα positive) were obtained from the American Type Culture Collection (Manassas, Virginia); A2780 (ERα negative) and A2780CP (ERα negative/cisplatin resistant) were kindly provided by Dr G. Peter Raaphorst (Ottawa Regional Cancer Center, Ottawa, Canada). TOV-21G (ERα negative) and TOV-112D (ERα negative) were kindly provided by Dr Anne-Marie Mes-Masson (Centre de Recherche du Center Hospitalier de L’Université de Montréal, Montréal, Canada). The Skov-3 cell line was maintained in McCoy’s medium containing 10% fetal growth serum and 50 µg/mL gentamycin; Ovar-3 was maintained in RPMI-1640 medium containing 10% fetal bovine serum and 50 µg/mL gentamycin; A2780 and A2780CP cell lines were maintained in DMEM-F12 medium containing 2% bovine growth serum and 50 µg/mL gentamycin; TOV-21G and TOV-112D were maintained in ovarian surface epithelial medium containing 10% fetal growth serum, 50 µg/mL gentamycin, and 0.5 µg/mL amphotericin B. The cells were maintained at 37°C with 5% CO₂.

**Antibodies and reagents**

All primary antibodies (see Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org) were obtained from Cell Signaling Technology (Beverly, Massachusetts) except for Erα (Ab-16 from Neomarkers, Thermo Fisher Scientific, Fremont, California) and loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, Massachusetts) and β-actin (Sigma, St Louis, Missouri). Secondary antibodies and horse-radish peroxidase-conjugated goat anti-rabbit was from Bio-Rad Laboratories (Mississauga, Ontario, Canada) and horseradish peroxidase-conjugated goat antimouse from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). Cisplatin and acridine orange were purchased from Sigma, the annexin V/PI apoptosis kit was purchased from Invitrogen (Burlington, Ontario, Canada), chloroquine diphosphate salt was purchased from Sigma, and the 17β-estradiol-platinum(II) hybrid VP-128 was synthesized using our most recent methodology and purified by chromatography [Descoteaux et al (26)].

**Drug treatments**

Skov-3, Ovar-3, A2780, and A2780CP cells were seeded in 100-mm² petri dishes (5 × 10⁵ Skov-3 cells, 5 × 10⁵ Ovar-3 cells, 3 × 10⁵ TOV-21G cells, 4 × 10⁵ TOV-112D cells, and 4.5 × 10⁵ A2780/A2780CP cells per dish). The following day, cells were pretreated or not with chloroquine diphosphate salt for 1 hour, followed by treatment with increasing concentrations of cisplatin or VP-128 (0, 1, 5 or 10 µM) for the indicated time periods (8 or 12 hours). Treatments were done in full estrogenic medium including phenol red and complete fetal bovine serum/gentamycin; 50 µg/mL gentamycin; and 0.5 µg/mL of selected plasmids.

**Measurement of annexin V/PI cells**

Fluorescein isothiocyanate annexin V/PI apoptosis kit (Molecular Probes Inc, Eugene, Oregon) was used according to the manufacturer’s instructions. Briefly, the treated cells were collected, washed with PBS, and then diluted in 1× annexin binding buffer (100 µL). For each sample, 5 µL of annexin V and 1 µL of propidium iodide were added to the cell suspension and then incubated 15 minutes at room temperature. After incubation time, an additional 100 µL of the annexin binding buffer was added to each sample for a total of 200 µL. Samples were analyzed (6000–10 000 events) using a Beckman Coulter flow cytometer FC500 (Beckman Coulter, Mississauga, Ontario, Canada). Analyses were performed using Kaluza software (version 1.1; Beckman Coulter).

**Transient transfection**

A2780 ovarian cancer cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and 4 µg of selected plasmids. pVP16-ERα (Addgene plasmid 11351) was kindly provided by Dr Donald P. McDonnell (29). Briefly, cells were transiently
transfected with pVP-empty or pVP-ER\textsubscript{α} plasmids using Lipofectamine 2000 (Invitrogen) for 6 hours, and the media were then replaced and cells were let to recover for 18 hours before harvesting.

Lentiviral transfection

Lentiviral particles were produced using the lenti-X HTX packaging system and human embryonic kidney-293T cells from CLONTECH Laboratories (Mountain View, California). MISON pLKO.1-puro nontarget short hairpin RNA (shRNA) control plasmid and 5 different MISON shRNA constructs targeting ER\textsubscript{α} from Sigma-Aldrich (St Louis, Missouri) were used for the production of the lentiviral particles. Skov-3 ovarian cancer cells were then stably transfected with supernatant containing lentiviral particles of various shRNA constructs for 24 hours, and the media were then replaced and the cells were let to recover for 24 hours. The selection of the cells was then made using puromycin at 0.75 \( \mu \)g/mL for 5 days.

Hoescht nuclear staining

The treated cells were collected, washed twice in PBS, suspended at an approximate density of 2 \( \times 10^5 \) cells/mL in PBS containing Hoechst 33258 (Sigma), and incubated for 24 hours at 4°C before fluorescence microscopy analysis of the apoptotic cells. At least 200 cells were counted for each sample, and a percentage of apoptotic cells was calculated as the ratio of apoptotic cells (with characteristic apoptotic morphology such as nuclear shrinkage and condensation) to total cell count.

RNA extraction and RT-PCR

Total RNA was isolated from cells using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 0.4 \( \mu \)g RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Primers for PCR amplification were as follows: 5′-GTGCGCTG-GCTAGAGATCCTG-3′ (sense) and 5′-AGAGACCTCAAG GTGCTTGGGA-3′ (antisense) for ER\textsubscript{α}; 5′-GAGGATCTTCAT GAGGTAGTCTGTCAGGTC-3′ (sense) and 5′-CACCTGGGA CGACATGAGAAGATCGGCA-3′ (antisense) for \( \beta \)-actin. PCRs were conducted in a MJ Research (Waltham, Massachusetts) thermal cycler (model PTC-100) using the following parameters: 30 seconds at 94°C, 30 seconds at 66°C, and 1 minute at 72°C for 40 cycles (ER\textsubscript{α}) and 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C for 25 cycles (\( \beta \)-actin). The reaction mixture was size separated on an agarose gel and visualised using SYBR-Safe (Invitrogen) staining upon UV transillumination.

Western blot

Treated cells were washed with PBS and submitted to lysis in cold radioimmunoprecipitation assay buffer containing protease inhibitors (Complete; Roche Applied Science, Laval, Québec, Canada) followed by 3 freeze–thaw cycles. Equal amounts of cell lysates, determined using a Bio-Rad DC protein assay (Bio-Rad Laboratories), were separated onto polyacrylamide gels (8%–15%) and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked in 5% milk, PBS 1\( \times \), 0.06% Tween 20 for 1 hour at room temperature, probed with a primary antibody, washed in PBS 1\( \times \), 0.06% Tween 20, and incubated with horseradish peroxidase-conjugated second-ary antibody (Bio-Rad Laboratories). Detection was performed using a SuperSignal West Femto substrate (Thermo Fisher Scientific, Nepean, Ontario, Canada), as described by the manufacturer using UVP bioimaging systems. Densitometry was done using Quantity One software, version 4.6.9 (Bio-Rad Laboratories).

Subcellular fractionation

NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific, Nepean, Ontario, Canada) was used according to the manufacturer’s instructions. The cytoplasmic proteins were collected using CERI and II reagents, whereas proteins from the nucleus were collected using the NER reagent. GAPDH was used as a loading/purity control for cytoplasmic proteins, whereas poly(ADP-ribose) polymerase (PARP) was used for nuclear proteins.

Immunofluorescence

Treated cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, rinsed in PBS, and then processed for immunostaining. Cells were blocked with protein block solution from Dako (Burlington, Ontario, Canada). Cells were then rinsed in PBS, incubated with a polyclonal antirabbit apoptosis-inducing factor (AIF) or negative control IgG (Vector Laboratories, Burlington, Ontario, Canada) for 1 hour, rinsed in PBS, and then incubated with the secondary antibody, donkey antirabbit IgG conjugated to Alexa Fluor 488 (Invitrogen) for 30 minutes. Cells were then incubated with rhodamine-phalloidin (Molecular Probes) for 20 minutes to stain actin microfilaments. The slides were washed once in PBS and counterstained with Hoechst nuclear dye for 5 minutes followed by 2 rinses in water. The sections were mounted in 0.1% P-phenylene diamine in 50% glycerol, and photomicrographs were taken with a Zeiss Axio Observer microscope (Carl Zeiss, Toronto, Canada).

Acridine orange

The treated cells were stained with 0.5 \( \mu \)g/mL acridine orange (Sigma) in complete medium at 37°C for 15 minutes. After the incubation, samples were collected and then analyzed (6000–10 000 events) using a Beckman Coulter flow cytometer Cytomics FC500. Analyses were performed using CXP software (Beckman Coulter).

In vivo experiments

All animal protocols were approved by the Université du Québec à Trois-Rivières Animal Care Committee. Subcutaneous tumor xenografts of human cancer cells were established in 6-week-old female CD-1 nude mice (Charles River Laboratories, Lasalle, Québec, Canada) by injection of 10 \( \times 10^6 \) cells in 100 \( \mu \)L of 2 mg/mL Matrigel (VWR, Mississauga, Ontario, Canada) at both flanks near the posterior legs. The mice used were not ovariectomized for these experiments. Starting on the day of xenografts, mice inoculated with hormone-dependent Ovcar-3 tumor cells received a sc injection of E2 (0.15 mg/animal) at 3-day intervals to prime tumor growth. These injections of E2 were continued once the treatments with anticancer drugs were initiated. For each animal, the tumor size was measured twice a week using calipers. The tumor volume was calculated using the following formula: \( 0.5 \times \text{length} \times \text{width}^2 \) (75).

Treatments with anticancer drugs were initiated when the tumors derived from a given cell line reached a mean volume of...
100 mm³ (day 0). Mice received ip injections of either VP-128 or cisplatin (0.00615 mmol/kg) or control (vehicle) solution (5% Cremophor (Sigma), 5% ethanol diluted in 0.9% sodium chloride), at 3-day interval starting at day 0. Animals were also weighed twice a week, starting at day 0. Mice were killed after 21 days; at that time, tumor size had not grown greater than 2500 mm³. Mice were subjected to macroscopic examination at the time of necropsy, but no detectable abnormality was noted.

**Statistical analyses**

The data were subjected to 1-way ANOVA (PRISM software, version 5.00; GraphPad, San Diego, California). Differences between experimental groups were determined by the Tukey’s test. Statistical significance was accepted when $P < .05$.

**Results**

**ERα enhances VP-128 activity in ovarian cancer cell line**

A2780 (ERα−) were transiently transfected with pVP16-ERα to overexpress the ERα. Skov-3 (ERα+) was stably transfected with lentiviral particles containing shRNA of ERα to knock down the ERα. Expression of ERα mRNA and protein compared with A2780 and Skov-3 wild type and empty vector confirmed the presence or absence of the ER after transfection (Figure 1, A and B). Analyses of Hoescht staining were performed to observe morphological changes of apoptotic cells induced by E2-Pt(II) hybrid (VP-128) and cisplatin in empty A2780 (ERα−) or Skov-3 (ERα+) and transfected A2780 (ERα+) or Skov-3 (ERα−) (Figure 1, A and B). We observed that VP-128 dramatically induced more apoptosis (more than 2-fold) in transfected A2780 (ERα+) than in empty A2780 (ERα−), showing a selectivity and improved efficiency toward cells possessing its target (ERα). Similar results happens in shRNA ERα number 4–5 Skov-3 (ERα−) in which VP-128 induced significantly less apoptosis (more than 2-fold) than in nontarget shRNA Skov-3 (ERα+). Cisplatin showed no significant increase.

**Figure 1.** Enhanced action of VP-128 after overexpression of ERα in the ovarian cancer cell line A2780 in vitro. A, A2780 cells were transiently transfected with pVP-empty or pVP-ERα plasmids using Lipofectamine 2000 (Invitrogen for 6 hours, media were then replaced, and cells were let to recover for 18 hours. Expression of ER-α, as evaluated by RT-PCR and Western blot analysis, on total RNA is shown, and protein was collected 24 hours after transfection. Transfected cells were seeded in 6-well plates and treated with VP-128 (1 μM) or cisplatin (5 μM) for 12 hours. Cis, cisplatin; Ctrl, control. B, Skov-3 cells were stably transfected with lentiviral particles containing shRNA of ERα. Expression of ERα, as evaluated by RT-PCR and Western blot analysis, on total RNA is shown, and protein was collected. Transfected cells were seeded in 6-well plates and treated with VP-128 (10 μM) or cisplatin (20 μM) for 24 hours. After corresponding treatment, cells were collected and stained with Hoescht 33258 for the evaluation of apoptosis induction by nuclear staining. Results are mean ± SEM of 3 independent experiments. *, $P < .05$ compared with the corresponding mock-treated cells; †, $P < .0001$ compared with the corresponding empty vector cells.
of apoptosis in the presence or absence of ERα, showing no selectivity using this drug. VP-128 is also more efficient than cisplatin to induce apoptosis in the presence or absence of its target (ERα). This is shown by the drug concentration required for VP-128 (1 μM) compared with cisplatin (5 μM) to induce more efficiently cell death in chemosensitive A2780(ERα−/+ ) ovarian cancer cells (Figure 1A). The same situation can be observed in chemoresistant Skov-3(ERα−/+ ) ovarian cancer cells in which 10 μM is used with VP-128 when compared with 20 μM used for cisplatin. Altogether these results suggest that VP-128 is more efficient against ovarian cancer cells possessing its target, ERα but is also effective against ERα-negative cancer cells, suggesting it may use different mechanisms of action than its base compound, cisplatin.

Enhanced antitumor activity using VP-128 in vivo toward hormone-dependent ovarian cancer xenografts when compared with its basic compound, cisplatin

Nude mice bearing hormone-dependent Ovcar-3 (ERα+) (30) or hormone-independent A2780 and A2780CP (ERα−) sc ovarian cancer cells xenografts were used to evaluate the efficacy of VP-128 to reduce tumor size in vivo. VP-128 administered to mice effectively suppressed the progression of hormone-dependent resistant derived from Ovcar-3 cells (Figure 2A) and hormone-independent sensitive derived from A2780 cells (Figure 2B) as well as hormone-independent resistant derived from A2780CP (Figure 2C) tumors in vivo, indicating that VP-128 can apply antitumor activity over the 3 types of ovarian cancer cells in vivo. Interestingly, VP-128 suppressed the progression of cisplatin-resistant tumors derived from Ovcar-3 (Figure 2A) and A2780CP cells (Figure 2C) more efficiently than cisplatin, indicating its competence against platinum-resistant tumors. It is also very interesting to notice that VP-128 efficiently reduced tumor size when compared with cisplatin only in the case of ERα+ cells (Ovcar-3, Figure 2A), demonstrating the increased efficacy and selectivity of the estradiol-platinum complex compared with cisplatin to treat hormone-dependent tumors. At the concentrations used, animals treated with VP-128 or cisplatin did not suffer any weight loss (Figure 2, A–C). No visible sign of toxic side effects were apparent, both during the administration of drug and upon examination of all organs at the time of necropsy. Treatment on nude mice bearing A2780CP cells (Figure 2C) was stopped on the 14th day because of the tumor size, which was too big for the mice.

Figure 2. Improvement of antitumor activity using VP-128 compared with its basic compound cisplatin toward ERα-positive chemoresistant ovarian cancer cells in vivo. ERα-positive chemoresistant ovarian cancer cell line Ovcar-3 (A), ERα-negative chemosensitive ovarian cancer cell line A2780 (B), and ERα-negative cisplatin resistant ovarian cancer cell line A2780CP (C) were inoculated sc in the flanks of CD-1 nude mice. When tumors reached a mean volume of 100 mm3 (day 0), mice received ip injections of either VP-128 or cisplatin (0.00615 mmol/kg) or control (mock treatment), at 3-day intervals. Tumor volume was measured routinely using calipers; tumor size at day 0 of treatment corresponds to 100%. Mice weight was determined twice a week; weight at day 0 corresponds to 100%. Results are mean ± SEM of the indicated number of mice per group. *, P < .01 VP-128 compared with control-treated and cisplatin-treated tumors; †, P < .0001 VP-128 compared with control-treated tumors only. Cispl, cisplatin; Ctl, control.
We also carried out a complete histological examination on the brain, heart, liver, lung, spleen, stomach, uterus, and ovary tissues that were processed by a pathologist at the Institute for Research in Immunology and Cancer (Montréal, Canada) according to standard procedures (ie, fixation, embedding, sectioning at 2–3 μm and regular hematoxylin and eosin staining) (data not shown). The only significant lesions that were found were a malignant lymphoma arising in the spleen with dissemination of lymphoma cells to the liver in animal 2 (normal control) and a mild to moderate renal tubule regeneration in animals 3 and 4 (cisplatin treated group). As for VP128, no significant tissue alteration could be observed. The finding of a spleen lymphoma in mice is not an unusual event because it is observed occasionally (31–33). Renal tubular regeneration is sometimes associated with aging, but presuming the mice were all the same age, it points to a role of cisplatin in the development of these lesions.

VP-128 induces apoptosis by activating caspase mechanisms

Cisplatin is known for activating caspase mechanisms to induce apoptosis in ovarian cancer cells (34). Our results showed that after only 12 hours of treatment, VP-128 induced caspase-3 and -9 cleavage/activation in all 6 model cell lines (Figure 3, A–F). TOV-21G and TOV-112D are ERα− ovarian cancer cells (data not shown), and we considered them chemosensitive because of the low dose required to induce caspase cleavage with chemotherapeutic drugs. VP-128 also induced PARP cleavage/degradation in all cell lines (Figure 3, A–F). These are all markers of caspase-dependent apoptosis. Cisplatin showed significant increase of cleaved caspase-3 and -9 and PARP levels only in chemosensitive A2780 cells (Figure 3C), which are sensitive to the cisplatin drug (35). Cisplatin did not significantly induce caspase cleavage in the other resistant cell lines (Figure 3, A, B, and D), prob-

Figure 3. VP-128 and cisplatin activate caspase-dependent mechanisms in ovarian cancer cells in vitro. Ovarian cancer cell lines, Ovcar-3 (ERα-positive) (A), Skov-3 (ERα-positive) (B), A2780/CP (ERα-negative) (C and D), TOV-21G (ERα negative) (E), and TOV112-D (ERα negative) (F), were treated with increasing concentrations of VP-128 or cisplatin (0, 1, or 10 μM) for 12 hours. The levels of apoptotic markers cleaved caspase-9 and caspase-3, and cleaved PARP were determined in treated cells using Western blot analysis. β-Actin was used as a loading control. Results shown are representative of 3 independent experiments.
ably because of the short treatment time (12 hours). Chemoresistant ovarian cancer cells Ovcar-3 (Figure 3A) only had a low level of cleaved caspase-9, whereas Skov-3 (Figure 3B) showed a low level of cleaved PARP when treated with 10 μM cisplatin. VP-128 was also efficient at 1 μM of VP-128 only in ovarian cancer cells A2780, TOV-21G, and TOV-112D (Figure 3, C, E, and F) because of their chemosensitivity to platinum compound when compared with the chemoresistant ovarian cancer cell lines. PARP cleavage is a direct action of cleaved caspases required for apoptosis (36, 37), and these results suggest that VP-128 induces apoptosis in a caspase-dependent manner and is more efficient than cisplatin to induce apoptotic cell death.

**VP-128 activates caspase-independent apoptosis in ERα− A2780 cells**

Cisplatin can also induce apoptosis in ovarian cancer cells by activating a caspase-independent pathway involving AIF (38). AIF induces apoptosis by its translocation from the mitochondria to the nucleus in which it induces chromatin condensation and DNA fragmentation (39). Only VP-128 induced an increase of total AIF levels in both Ovcar-3 (ERα+) (Figure 4A) and A2780 (ERα−) (Figure 4B) cells after 8 hours of treatment. However, VP-128 induced AIF translocation to the nucleus only in A2780 (ERα−) (Figure 4B), suggesting that VP-128 triggers AIF translocation in ERα− ovarian cell lines to induce apoptosis using a complementary pathway. We also have verified by treating our cells up to 12 hours and no translocation occurred in Ovcar-3 (ERα+) (data not shown). It is also important to consider that PARP is also cleaved with VP-128 in A2780 (ERα−).

**VP-128 induces autophagy in ovarian cancer cells**

Autophagy occurs by the formation of autophagosome and then by the fusion with lysosome leading to acidic vesicular organelles (AVOs), called autophagolysosomes, which is a common characteristic of the autophagic cell (40). We used acridine orange, a lysosomotropic agent capable of staining AVOs, to rapidly quantify induction of autophagy (41). We stained ovarian cancer cells treated with VP-128 and cisplatin and analyzed the expression of red fluorescence, quantifying AVOs, by flow cytometry. Results showed that red fluorescence (FL3) increased in Ovcar-3 and Skov-3 (ERα+) (Figure 5, A and B) after 12 hours of treatment. Red fluorescence also increased after 8 hours in A2780 and A2780CP (ERα−) (Figure 5, C and D), indicating an increase of AVOs, meaning induction of autophagy. No significant increase of red fluorescence was observed after 12 hours in A2780 and A2780CP (ERα−) (data not shown) because of the high level of apoptosis present at this time of the treatment compared with the resistant cell lines Ovcar-3 and Skov-3 (ERα+) (Figure 5, A and B).

After this analysis, we observed the expression of Beclin-1 and Light Chain 3-B (LC3B) proteins. Beclin-1 and

![Figure 4](https://academic.oup.com/endo/article-fig/154/7/2281/2423162/1547228124337d2)
LC3B are proteins related to the formation of autophagosome, leading eventually to autophagy (42). LC3B-I is related to the formation of the autophagosome by converting its LC3B-I form to LC3B-II through lipidation of the protein. Our results showed that the Beclin-1 protein level decreased lightly after VP-128 treatment in some cell lines but not significantly (Figure 6, A–D). Nonetheless, VP-128 highly increased the level of LC3B-II for all cell lines (4–25 times) (Figure 6, A–D), indicating the formation of autophagosome and leading to autophagy. The LC3B-II increase correlated with the decrease of the LC3B-I form in all ovarian cancer cells treated with VP-
128 (Figure 6, A–D). The conversion of LC3B-I to LC3B-II after treatment with VP-128 suggests that autophagy is induced in these ovarian cancer cells treated with VP-128 and is in agreement with the formation of AVOs (Figure 5).

**AKT and mammalian target of rapamycin (mTor) pathways are involved in VP-128 induced-autophagy**

It has been reported previously that AKT and mTor pathways regulate negatively autophagy (43). mTor regulates it directly, whereas the phosphatidylinositol 3-kinase/AKT pathway is an upstream regulator of mTor (44). We decided to analyze these regulators of autophagy by Western blot (Figure 7). All ovarian cancer cell lines treated with VP-128 showed decrease of phosphorylated (active) AKT and mTor (Figure 7, A–D). Ovcar-3 and Skov-3 cancer cells did not showed a significant decrease of phosphorylated (p)-AKT (Figure 7, E and F), probably because of their resistance to chemotherapy on a short time period. The total AKT level is also decreased, suggesting that it consequently decreases the p-AKT level (Figure 7, A–D). Total mTor decreased in Skov-3, A2780, and A2780CP (Figure 7, B–D), again suggesting its relation with a decrease of p-mTor. Down-regulation of these pathways correlated with the induction of autophagy by VP-128 in ovarian cancer cells. Cisplatin did not decrease the level of AKT and mTor proteins, also suggesting that VP-128 may act faster than cisplatin (45, 46).

**Inhibition of autophagy improves VP-128 efficiency**

Autophagy occurring in cancer cells may induce either cytoprotection or cell death, depending on various factors (47). Our results showed that autophagy is induced in ovarian cancer cells treated with VP-128 (Figures 5–7), and we wanted to know whether the autophagy induced by our hybrid is promoting either cell survival or cell death. We decided to use chloroquine diphosphate salt, a lysosomotropic agent capable of inhibiting autophagy of cancer cells. By inhibiting autophagy via pretreatment of cells with chloroquine, we observed an increase of 10%–20% apoptosis induced to Skov-3 cells treated for 12 hours (Figure 8A) or A2780 cells treated for 8 hours (Figure 8B). This increase of apoptosis after inhibition of autophagy indicates a chemoprotective effect of autophagy induced by VP-128. To further investigate, we also looked at markers of apoptosis, cleaved caspase-3 and -9 and cleaved PARP, by Western blot analysis (Figure 8, C and D). We can observe a light increase of these markers when we pretreated cancer cells with chloroquine in combination with VP-128. To confirm induction of autophagy by VP-128 and its inhibition by chloroquine, we analyzed LC3B-II by Western blot analysis (Figure 8, C and D). Without using chloroquine, only cells treated with VP-128 showed an increase of LC3B-II. By doing pretreatment with chloroquine, accumulation of LC3B-II is observable in mock, cisplatin, and VP-128 treated ovarian cancer

![Figure 6](https://academic.oup.com/endo/article/154/7/2281/2423162)

**Figure 6.** VP-128 activates autophagosome mechanisms in ovarian cancer cells in vitro. Ovarian cancer cell lines, Ovcar-3 (A), Skov-3 (ERα-positive) (B), and A2780CP (ERα-negative) (C and D), were treated with increasing concentrations of VP-128 or cisplatin (0, 1, or 10 μM) for 12 hours. The levels of autophagosome-specific proteins Beclin-1 and LC3B-I/II were determined in treated cells using Western blot analysis. β-Actin was used as a loading control. Results shown are representative of 3 independent experiments. E–H, Densitometry of LC3B-II normalized with β-actin on ovarian cancer cell lines. Results are mean ± SEM of 3 independent experiments. *, P < .05 compared with corresponding mock-treated cells.
cells, indicating its incapacity to degrade autophagosomes and thus confirming the inhibition. Chloroquine inhibits autophagy at this late phase after the formation of autophagosomes by targeting lysosomal degradation, explaining the accumulation of LC3B-II in the Western blotting experiment (48–50).

Discussion

Our previous study revealed that VP-128 has selectivity toward hormone-dependent breast cancer cell lines and higher antitumor activity in vitro and in vivo than its reference compound, cisplatin (28, 51). In this study, we have investigated whether VP-128 was also efficient and more potent than cisplatin against another potential hormone-dependent cancer, ovarian cancer. We chose to use 10 μM of cisplatin based on previous publications from our laboratory (28, 52, 53). To better compare the effect of VP-128 with its base compound, cisplatin, we decided to use the same molar concentration during treatments, considering the presence of a single coordinated platinum molecule in both compounds. We have verified the antitumor...
activity of VP-128 in vitro, and our hybrid was once again more efficient than cisplatin to induce apoptosis in ERα+ but also in ERα− ovarian cancer cell lines. We have previously shown that VP-128 has more affinity for ERα than cisplatin (26), leading to selectivity toward hormone-dependent cancer cells such as breast cancer cells (28). Indeed, the transiently transfected ERα+ ovarian cancer cell line A2780 has shown an increase in the apoptotic index in response to VP-128 compared with cisplatin. The same event happens in the SKOV-3 shRNA ERα (ERα−) ovarian cancer cell line in which VP-128 apoptotic activity decreases more than 2-fold when its target (ERα) is knocked down. This high efficiency in the presence of our drug target (ERα) might be explained by the use of ERα, which translocates to the nucleus once linked with the E2 ligand (9, 10). Transportation of the drug to the nucleus using the E2 ligand improves VP-128 ability to bind to DNA and also activate proliferation mechanisms, then leading to apoptosis because of the platinum portion (14, 28). However, in the absence of its target (ERα) in empty ERα− ovarian cancer cell lines A2780 and SKOV-3 shRNA ERα, VP-128 is less efficient but is still active, and this may be a consequence of an easier diffusion of VP-128 through the cell membrane because of the presence of a steroid moiety (54, 55). By passing through the membrane, the VP-128 acts just like cisplatin as a normal platinum compound without binding to the ERα.

Another possible explanation is the presence of ERβ in A2780 ovarian cancer cells (56). Indeed, the estrogen ligand part of our hybrid also has attraction for the receptor sites on ERβ (26). However, it is important to consider that hormone-dependent cancer mainly overexpresses ERα, which is our primary target (4, 6, 7). Overall, VP-128 was more potent than cisplatin to induce apoptosis in ovarian cancer cells in vitro, and the presence of our target (ERα) greatly improved its efficiency.

We have also tested VP-128 antitumor activity in vivo using xenografts from ovarian cancer cells (Ovcar-3 ERα+, A2780 ERα− and A2780CP ERα−). Five milligrams per kilogram is a typical dose of cisplatin used for treating tumors in nude mice (57–59), and we decided to convert it into millimoles per kilogram because of the different molecular weight that cisplatin (300.06 g/mol) and VP-128 (812.33 g/mol) does have to better compare their...
efficiency in vivo. The concentration of 0.00615 mmol/kg was also the dose used in our previous publication of VP-128 on breast cancer (28). VP-128 and cisplatin were able to similarly stop the growth of xenografts from sensitive ERα− ovarian cancer cells, A2780. Growth of xenografts from resistant ERα− ovarian cancer cells A2780CP were stopped more efficiently with VP-128 when compared with cisplatin. Again, VP-128 was effective against cisplatin resistance, even in vivo. More interestingly, contrary to cisplatin, VP-128 was strong enough to significantly lower tumor size of ERα+ ovarian cancer cells, Ovcar-3. No additional side effect, when compared with cisplatin at the same concentration, was observed following the idea of lowering side effects of chemotherapy via targeting toward cancer cells.

This improved antitumor activity toward ERα+ xenografts with VP-128, compared with cisplatin, supports the idea of a ERα-dependent mode of action, which is effective against hormone-dependent tumors because it was previously demonstrated for hormone-dependent breast cancer xenografts (28). High selectivity toward ERα+ cancer cells in vivo and less selectivity in vitro might be associated with E2-induced proliferation mechanisms from endogenous E2 in ERα+ xenografts (60, 61). ERα+ xenografts are estrogen dependent, and VP-128 might compete for its receptor because of the 17β-estradiol portion of the drug. Contrary to ERα+ xenografts, ERα− xenografts do not require E2-induced proliferation, and then VP-128 does not compete with E2 for the receptor. It is also important to take into consideration cancer cell resistance to cisplatin in which A2780 (ERα−) is chemosensitive and A2780CP(ERα−) and Ovcar-3 (ERα+) are chemoresistant. VP-128 was still capable of reducing the tumor size of cisplatin-resistant Ovcar-3 (ERα+)+ xenografts, stopping tumor growth of cisplatin-resistant A2780CP (ERα−) more efficiently than cisplatin, and stopping the growth of the cisplatin-sensitive A2780 (ERα−) similarly than cisplatin, suggesting again selectivity toward hormone-dependent ovarian cancer and capacity to overcome cisplatin resistance. Nonetheless, the antitumor activity of VP-128 was improved in ERα+ ovarian cancer cells in vivo.

Noteworthy, VP-128 seemed to be able to overcome cisplatin resistance in the 3 resistant cancer cell lines studied (Ovcar-3, Skov-3, and A2780CP) in vitro by inducing the high level of cleaved caspase-3, caspase-9, and PARP long before cisplatin. The same thing occurs in vivo by reducing the tumor size efficiently in hormone-dependent Ovcar-3 xenografts (ERα+) and being more efficient than cisplatin with A2780CP xenografts (ERα−). In the literature, it has been shown that cisplatin resistance might be related to the exportation of the drug outside the cells via efflux pumps and membrane transporters (62, 63). The resistant ovarian cancer cell line A2780CP has an increased efflux rate of cisplatin related to the increased protein concentration of ATPase transporter (ATP7A and ATP7B) (62). The addition of a steroid portion (E2) on our hybrid, VP-128, makes our drug larger, and this may interfere with the efflux pumps mechanism by being unable to be exported outside the resistant ovarian cancer cells.

We have studied previously whether the estradiol-platinum(II) hybrid family interacts with DNA similarly to cisplatin. We confirmed that the hybrid could bind DNA duplex by the H-bonding network and guanine N7 sites similarly to cisplatin (64). We also made a preliminary pharmacokinetics experiment in vivo in our laboratory, and the results showed that VP-128 seemed to be stable and was cleared similarly to cisplatin drug after analyzing blood samples at different times (0–6 hours) after the administration of cisplatin/VP-128 to nude mice (data not shown). To further examine the VP-128 mechanism of action, we decided to study its ability to induce caspase-dependent apoptosis. After only 12 hours of treatment, VP-128 could already activate caspase pathways (Cl.Casp 3, Cl.Casp 9, and Cl.Parp), whereas cisplatin only induced a slight increase of these apoptotic markers (65). This indicates that VP-128 had a faster rate of apoptosis induction than cisplatin, using the same concentration for both drugs. VP-128 acts differently from cisplatin by inducing caspase-independent apoptosis in ERα− breast cancer cells by releasing AIF from the mitochondria and then translocating it to the nucleus (28). Again, in ovarian cancer cells, releases of AIF and accumulation to the nucleus have occurred only in the ERα− cell line, A2780, after treatment with VP-128, whereas cisplatin did not. Because our hybrid can bind ERβ with good affinity (26) and that ERα− cancer cell lines (A2780) have a high ratio of ERβ over ERα, the estradiol portion of our hybrid might contribute to the release of AIF (66, 67) to induce caspase-independent apoptosis. This shows that VP-128 acts via different apoptosis mechanisms in the presence and the absence of ERα.

Our present study indicates that VP-128 also induced autophagy in ovarian cancer cells. An increase in AVOs and the LC3B-II form occurred after treating cancer cells with VP-128, indicating the formation of the autophagosome, which leads to autophagy (42). We have also investigated another marker of autophagy, Beclin-1, which seemed to be slightly down-regulated after treatment with VP-128. Beclin-1 is a protein responsible for the initiation of autophagosome formation involved earlier than LC3B in the process of autophagy (68). It has been previously shown that autophagy is one of the initial responses of cancer cells under survival conditions. Severe stress in-
duced to the cells will then inhibit autophagy by cleaving proteins such as Beclin-1, leading to apoptosis (69, 70). The results obtained suggest that Beclin-1 might be cleaved by caspases such as caspase-3 (71) after 12 hours of treatment on cancer cells. Caspase-3 was cleaved after 12 hours of treatment following this hypothesis, suggesting that VP-128 induces autophagy followed by apoptotic cell death in ovarian cancer cells.

We finally explored pathways related to autophagy directly (mTor) and indirectly (AKT) to verify whether these were regulated negatively to induce autophagy (43). Our results showed that these pathways were inhibited in almost all ovarian cancer cell lines favoring autophagy induction. Including our hybrid VP-128, several pharmaceuticals drugs induce high amounts of autophagy in cancer cells in vitro, including ERα antagonist tamoxifen (72) and our base compound cisplatin (17). Autophagy induced by these antitumor compounds thus favor chemoresistance in cancer cells (73). We confirmed that this theory correlates with VP-128 actions because we found out by inhibiting autophagy that VP-128 cytotoxicity was improved, indicating the chemoprotective effect of autophagy. Autophagy inhibition sensitizes cancer cells, and many publications are showing improvement of drug efficiency when combined with an inhibitor of autophagy such as chloroquine (20, 74). Apoptosis and autophagy mechanisms both can be activated by similar stressor such as chemotherapeutics drugs. By activating both of these processes, some kind of inhibitory cross talk occurs against each other, and 1 of these 2 mechanisms will dominate (47). Thus, using an autophagy inhibitor in combination with VP-128 could be used to our advantage to sensitize ovarian cancer cells and increase apoptosis.

In conclusion, the ability of the VP-128 to overcome chemoresistance and to surpass cisplatin for the treatment of hormone-dependent (ERα+) ovarian cancer cells in vivo and in vitro demonstrates the interesting potential of our E2-platinum(II) hybrid as an anticancer agent. The fact that VP-128 does not increase cytotoxicity in vivo when compared with its reference compound, cisplatin (17), indicates that VP-128 is not inducing apoptosis but also autophagy. Combining VP-128 with an autophagy inhibitor could increase the benefit of this new and selective chemotherapeutic hybrid. This study demonstrates the potential of VP-128 to be used with many hormone-dependent feminine cancers (28).

Acknowledgments

We thank Gabriel Chateauneuf for technical assistance. C. Descoteaux of the Natural Sciences and Engineering Research Council of Canada scholarship is also gratefully acknowledged.

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This work was supported by a team grant from the Fonds Québécois de la Recherche sur la Nature et les Technologies and a grant from the Canadian Institute of Health Research. E.A. is the chairholder of the Canada Research Chair in Molecular Gynec-oncology. K.B. was the holder of a master scholarship from the Réseau Québécois en Reproduction, the Fonds de la Recherche en Santé du Québec, and the Canadian Institute of Health Research.

Disclosure Summary: The authors have no conflict of interest to declare.

References

17. Fanzani A, Zanola A, Rovetta F, Rossi S, Aleo MF. Cisplatin triggers atrophy of skeletal C2C12 myotubes via impairment of Akt signal-


