Lurbinectedin (PM01183), a New DNA Minor Groove Binder, Inhibits Growth of Orthotopic Primary Graft of Cisplatin-Resistant Epithelial Ovarian Cancer

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Abstract

Purpose: Epithelial ovarian cancer (EOC) is the fifth leading cause of death in women diagnosed with gynecologic malignancies. The low survival rate is because of its advanced-stage diagnosis and either intrinsic or acquired resistance to standard platinum-based chemotherapy. So, the development of effective innovative therapeutic strategies to overcome cisplatin resistance remains a high priority.

Experimental Design: To investigate new treatments in in vivo models reproducing EOCs tumor growth, we generated a preclinical model of ovarian cancer after orthotopic implantation of a primary serous tumor in nude mice. Further, matched model of acquired cisplatin-resistant tumor version was successfully derived in mice. Effectiveness of lurbinectedin (PM01183) treatment, a novel marine-derived DNA minor groove covalent binder, was assessed in both preclinical models as a single and a combined-cisplatin agent.

Results: Orthotopically perpetuated tumor grafts mimic the histopathological characteristics of primary patients’ tumors and they also recapitulate in mice characteristic features of tumor response to cisplatin treatments. We showed that single lurbinectedin or cisplatin-combined therapies were effective in treating cisplatin-sensitive and cisplatin-resistant preclinical ovarian tumor models. Furthermore, the strongest in vivo synergistic effect was observed for combined treatments, especially in cisplatin-resistant tumors. Lurbinectedin tumor growth inhibition was associated with reduced proliferation, increased rate of aberrant mitosis, and subsequent induced apoptosis.

Conclusions: Taken together, preclinical orthotopic ovarian tumor grafts are useful tools for drug development, providing hard evidence that lurbinectedin might be a useful therapy in the treatment of EOC by overcoming cisplatin resistance. Clin Cancer Res; 18(19); 5399–411. ©2012 AACR.

Introduction

Ovarian cancer is the fifth leading cause of death among women, and is the most common cause arising from gynecologic malignancies (1). Although progress has been made in the treatment of epithelial ovarian cancer (EOC) by improved surgical debulking and the introduction of platinum-taxane regimens, overall 5-year survival rate is only 29% in advanced-stage disease (2–6). This low survival rate is because of its frequent diagnosis at an advanced stage and by intrinsic and acquired resistance to platinum-based chemotherapy. In the recurrent disease setting, those patients who experience progression through first-line, platinum-based therapy (platinum refractory), or those who experience relapse within 6 months of receiving platinum therapy (platinum resistant) are typically treated with a second-line non–platinum-based regimen, such as single-agent doxorubicin (7) gemcitabine (8), paclitaxel,
Several lines of evidence indicate that engrafting primary tumor tissues orthotopically into immune-deficient mice (termed "tumor grafts") may be outstandingly valuable preclinical models for new drug development, and for reducing the high failure rate that exists in translating preclinical results to patients (29-33).

Here, we report the establishment and characterization of a serially transplantable, orthotopic, subject-derived epithelial ovarian tumor graft that retains crucial characteristics of the original primary tumor specimen, and its further development as an in vivo cisplatin-resistance tumor model. We show in engrafted preclinical models that lurbinectin, a new minor groove DNA binder, is effective in the treatment of experimental ovarian tumors as a single or a combined-cisplatin agent. Overall, we present evidence of the efficacy of a therapeutic strategy based on the idea that a combination of 2 drugs that bind differentially to each DNA groove could overcome frequent cisplatin resistance in advanced-stage ovarian cancer.

Materials and Methods

Drugs and cell lines

Lyophilized lurbinectin (PM01183) vials (1 mg/mL) were obtained from PharmaMar (Colmenar Viejo) and cisplatin (1 mg/mL) from Ferrer-Farma. The A2780 human ovarian carcinoma cell line was obtained from the European Collection of Cell Cultures. Cell cultures were grown in vitro at 37 °C in a humidified atmosphere of 5% CO₂ in RPMI-1640 (Sigma-Aldrich Co.) supplemented with 10% FBS.

Animals

Female athymic nu/nu mice (Harlan) between 4 to 6 weeks of age were housed in individually ventilated cages on a 12-hour light-dark cycle at 21 to 23 °C and 40% to 60% humidity. Mice were allowed free access to an irradiated diet and sterilized water. All animal protocols were reviewed and approved according to regional Institutional Animal Care and Use Committees.

Primary sample and orthotopic tumor engrafted in mice

The primary tumor specimen was obtained at Hospital Universitari de Bellvitge (L’Hospitalet de Llobregat, Barcelona, Spain). The study was approved by the Institutional Review Board. Written informed consent was collected from a patient who had not received cisplatin-based chemotherapy. Nonnecrotic tissue pieces (ca. 2–3 mm³) from resected serous human epithelial ovarian tumor were selected and placed in DMEM (BioWhittaker) supplemented with 10% FBS and penicillin/streptomycin at room temperature. Under isofluorane-induced anesthesia, animals were subjected to a lateral laparotomy, their ovaries exposed and tumor pieces anchored to the ovary surface with prolene 7.0 sutures. Tumor growth was monitored 2 to 3 times per week and when the tumor grew, it was harvested, cut into small fragments, and transplanted into 2 to 5 new animals. Engrafted tumors at early mouse passages were cut in 6 to 8 mm³ pieces and stored in liquid nitrogen for a solution of preclinical models in drug testing, we have developed two paired (cisplatin-sensitive and cisplatin-resistant) preclinical models of serous carcinoma phenocopying patients’ primary tumor features including chemotherapy response behavior. In this study, we show that single lurbinectin (PM01183), a novel marine-derived DNA minor groove covalent binder, or cisplatin-combined therapies were effective in treating cisplatin-sensitive and cisplatin-resistant preclinical models. Thus, we present hard evidences that lurbinectin might be a useful therapy in epithelial ovarian cancer overcoming acquired cisplatin resistance providing a rationale for future trials.

DNA structure features 2 well-defined clefts known as the major and minor grooves, and DNA-binding proteins and drugs usually make contacts with the sides of the bases exposed in both grooves (16, 17). The DNA major groove is a site of attack for cisplatin and many alkylating agents, and when cisplatin binds to DNA 3 types of lesions can be formed on purine bases: monoadducts, and intra- and interstrand crosslinks. On the other hand, other antitumor drugs including mitomycin C, chromomycin A₃, and eteincasidins, bind to the minor groove (18). One of the best examples is trabectedin (Yondelis), which reacts with certain guanines in the minor groove of DNA to form a covalent bond (19–21). The adduct is stabilized by van der Waals interactions with nucleotides in the opposite DNA strand, creating the equivalent of a functional interstrand crosslink (22). Lurbinectin (PM01183) is a new synthetic alkaloid that is structurally related to eteincasidins (23), which, with the exception of a module addition (ring C), confer important pharmacokinetic and pharmacodynamics properties benefits as well intrinsic activity (24–26).

Establishment of preclinical models phenocopying patients’ primary tumor features, which accurately reflect phenotypic, genotypic, and tumor chemotherapy response behavior, is a basic step on the way to identifying novel therapeutic targets and for testing novel treatments (27, 28). Several lines of evidence indicate that engrafting primary tumor tissues orthotopically into immune-deficient mice could overcome frequent cisplatin resistance in advanced-stage ovarian cancer.

Translational Relevance

The efficacy of conventional platinum-based chemotherapy for EOCs is limited; most patients show an initial response to treatment but upon relapse, the platinum response rates progressively diminish and they ultimately die. So, the development of effective innovative therapeutic strategies to overcome cisplatin resistance remains a high priority. On the way to identifying novel therapeutic targets and for drug testing, we have developed two paired (cisplatin-sensitive and cisplatin-resistant) preclinical models of serous carcinoma phenocopying patients’ primary tumor features including chemotherapy response behavior. In this study, we show that single lurbinectin (PM01183), a novel marine-derived DNA minor groove covalent binder, or cisplatin-combined therapies were effective in treating cisplatin-sensitive and cisplatin-resistant preclinical models. Thus, we present hard evidences that lurbinectin might be a useful therapy in epithelial ovarian cancer overcoming acquired cisplatin resistance providing a rationale for future trials.
90% FBS and 10% dimethyl sulfoxide for subsequent implantation.

**Histology and immunohistochemical tumor characterization**

The morphology of the primary patient’s tumor and of the both engrafted tumors (OVA1X and OVA1XR) was compared by H&E staining in paraffin-embedded sections. Determination of cytokeratin (CK) 7, Ki67, WT1, alpha estrogen, and progesterone receptors status by immunohistochemistry, in accordance with the standard clinical protocols of the Department of Pathology (see Fig. 1 legend).

**In vivo establishment of cisplatin-resistant xenografted tumor**

Cisplatin-resistant tumor (named OVA1XR) was developed by iterative cycles of in vivo exposure to cisplatin of OVA1X. Briefly, orthotopically engrafted OVA1X tumors (at mouse passage #3) were allowed to grow until intra-abdominal palpable masses were noted. Then, animals were intravenously (i.v.) administered with cisplatin at a dose of 2 mg/kg for 3 consecutive weeks (Days 0, 7 and 14; cycle#1 of treatment). Post-cisplatin tumor relapse were harvested, prepared as previously described, and engrafted in new animals. This process was repeated up to five times by treating tumor-bearing mice with stepwise increasing doses of cisplatin: cycle#2, 3 mg/kg; cycle#3, 3.5 mg/kg; cycle#4, 4 mg/kg; and cycle#5, 5 mg/kg (see Fig. 1C). Cisplatin-resistant tumors were obtained in three independent experiments (OVA1XR-L1, -L2 and -L3). At doses higher than 3.5 mg/kg, signs of cisplatin-induced toxicity were ameliorated by 2 days administration of saline containing 5% glucose.

**Drug treatment of engrafted cisplatin-sensitive and cisplatin-resistant tumor models**

Mice were transplanted with fragments of OVAX1 and OVAX1R tumors, and when tumors reached a homogeneous palpable size were randomly allocated to the treatment groups (n = 8–12/group): i) Placebo; ii) Lurbinectedin (0.18 mg/kg); iii) Cisplatin (3.5 mg/kg); and iv) Lurbinectedin plus cisplatin (0.18 + 3.5 mg/kg). Drugs were i.v. administered once per week for 3 consecutive weeks (days 0, 7, and 14). Seven days after the final dose (day 21), animals were sacrificed, their ovaries dissected out, and weighed. Representative fragments were either frozen in nitrogen or fixed and then processed for paraffin embedding.

**Evaluation of histologic response after chemotherapy treatment**

Regressive histopathological features were evaluated (34–37), and 3 histologic response categories were established (38): (i) NHR, no histopathological response (<1 regression criterion [+] present); (ii) MHR, moderate (2 regression criteria [+] present); and (iii) GHR, good histopathological response (≥3 regression criteria [+] present).

**In vivo evaluation of synergism among lurbinectedin and cisplatin treatments**

Female mice were subcutaneously implanted with 10⁷ A2780 cells suspended in a 1:1 solution of RPMI-1640: Matrigel (Becton, Dickinson & Co.). Mice bearing tumors (ca. 150 mm³) were randomly allocated to 13 treatment groups (see Fig. 3 legend). All treatments were intravenously administered once per week for 2 consecutive weeks (days 0 and 7). Tumor growth was recorded 2 to 3 times per week starting from the first day of treatment (day 0) and tumor volume (in mm³), estimated according to the formula $V = (a \cdot b^2)/2$, (a: length or biggest diameter; $b$: width or smallest diameter). Antitumor drug activity was measured with respect to the $T/C$ index, and the fraction affected ($F_a$) by treatment was calculated ($F_a = 1−T/C$). A CI was determined by the CI-isobol method (39).

**Determination of tumor proliferation, apoptosis, and angiogenesis**

Proliferation was assessed by quantifying the anti-phospho-Histone H3 (S10; Millipore) mitosis marker as described (40). Aberrant mitotic figures were identified by double immunostaining with $\alpha$-tubulin (1:200) and anti-phospho-Histone H3 (S10; ref. 40). Apoptotic cells were quantified with two approaches: (i) immunostaining in paraffin-embedded samples with anti-Cleaved Caspase-3 (Asp175) antibody (Cell Signaling) at 1:200; and (ii) by terminal deoxynucleotidyl transferase-mediated biotin-diUTP nick end labeling (TUNEL) staining kit (Promega) in frozen OCT tissues (41).

**Statistical analysis**

Postchemotherapy tumor weight data were analyzed using a 2-tailed Mann–Whitney U test. The data are presented as medians and interquartile ranges (IQR) or means ± SD. Statistical analyses were done and graphs plotted using GraphPad Prism, version 5.02 (GraphPad Software Inc.). Synergism analyses were done by CompuSyn, version 1.0 (ComboSyn Inc.).

**Results**

**Orthotopic model of epithelial ovarian cancer mimics the histopathological characteristics of primary patients’ tumors**

Primary tumors engrafted in the ovarian surface of athymic female mice (named OVA1X) grew as large solid masses. Ovarian infiltration and neighboring organ invasion were not seen in any of the implanted animals (Fig. 1A). The engrafted rate was close to 95% in all mouse-to-mouse passages, with a mean time of ca. 1,000 to 1,500 mm³ during the first 6 passages of 84 ± 8 days. As shown in Fig. 1A, a very high histologic correlation was found between primary and engrafted tumors. Indeed, OVA1X had a typical serous adenocarcinoma appearance showing high cellularity, cellular papillae formation, and irregular slit-like spaces, and it remained stable throughout multiple rounds of serial mouse-to-mouse transplantation.
Figure 1. Establishment, and comparative histopathological characterization of primary and engrafted OVA1X and its paired developed cisplatin-resistant OVA1XR tumor. A, top, lateral laparatomy was conducted in isofluorane-anesthetized mice, the ovary mobilized and small tumor pieces of primary tumor anchored on the ovarian mouse surface with prolene 7.0 sutures. Engrafted tumors grew as large solid masses (usually 1,000–1,500 mm³) in diameter at the time of sacrifice, and ovarian infiltration/invasion or ascitis were not seen. Bottom, representative H&E and immunohistochemical staining reveals a high correlation between primary and paired engrafted tumors. Primary antibodies were monoclonal antibodies: CK7 (clone OV-TL 12/30, Dako); Ki67 (clone MIB-1, Dako); WT1 (clone 6F-H2, Dako) and estrogen receptor alpha (clone SP1, Dako) OV, ovary; TL, tumor engrafted in the left ovarian; TD, engrafted in the right ovarian; UT, uterus. B, mice engrafted with OVA1X tumor were treated intravenously with low (2 mg/kg), intermediate (3.5 mg/kg), and high (5 mg/kg) cisplatin doses, and either short- or long-term responses were observed.

Cycles of treatment

Postchemotherapy relapsed tumor was engrafted in the ovaries of other young mice, and this cycle repeated up to 5 additional times. Increased doses of cisplatin were used through the process: cycle #2: 3 mg/kg; cycle #3: 3.5 mg/kg; cycle #4: 4 mg/kg and cycle #5 and successive cycles: 5 mg/kg.

A, top, lateral laparatomy was conducted in isofluorane-anesthetized mice, the ovary mobilized and small tumor pieces of primary tumor anchored on the ovarian mouse surface with prolene 7.0 sutures. Engrafted tumors grew as large solid masses (usually 1,000–1,500 mm³) in diameter at the time of sacrifice, and ovarian infiltration/invasion or ascitis were not seen. Bottom, representative H&E and immunohistochemical staining reveals a high correlation between primary and paired engrafted tumors. Primary antibodies were monoclonal antibodies: CK7 (clone OV-TL 12/30, Dako); Ki67 (clone MIB-1, Dako); WT1 (clone 6F-H2, Dako) and estrogen receptor alpha (clone SP1, Dako) OV, ovary; TL, tumor engrafted in the left ovarian; TD, engrafted in the right ovarian; UT, uterus. B, mice engrafted with OVA1X tumor were treated intravenously with low (2 mg/kg), intermediate (3.5 mg/kg), and high (5 mg/kg) cisplatin doses, and either short- or long-term responses were observed.

B

OVA1X

Tumor weight, gr

Placebo (n = 10)

2 mg/kg cisplatin (n = 10 Short-term response; n = 10 Long-term response)

3.5 mg/kg cisplatin (n = 10 Short-term; n = 10 Long-term response)

5 mg/kg cisplatin (n = 10 Short-term; n = 10 Long-term response)

Placebo (n = 10)

2 mg/kg cisplatin (n = 10)

3.5 mg/kg cisplatin (n = 10)

5 mg/kg cisplatin (n = 10)

Tumor weight, gr

OVA1XR-L1 OVA1XR-L2 OVA1XR-L3

Cycle #1

Time lag between treatment and tumor passage, d

Cycles of cisplatin treatment in different mice

placebo

2 mg/kg cisplatin (n = 10)

3.5 mg/kg cisplatin (n = 10)

5 mg/kg cisplatin (n = 10)

OVA1X OVA1XR-L1 OVA1XR-L2 OVA1XR-L3

Placebo (n = 10) 2 mg/kg of cisplatin (n = 10)

3.5 mg/kg of cisplatin (n = 10)

5 mg/kg of cisplatin (n = 10)

Placebo (n = 10) 2 mg/kg of cisplatin (n = 10)

3.5 mg/kg of cisplatin (n = 10)

5 mg/kg of cisplatin (n = 10)

Placebo (n = 10) 2 mg/kg of cisplatin (n = 10)
Ki-67 immunostaining revealed a similar proliferative rate in primary and engrafted tumors, and they both preserved the same cytokeratin 7 (CK7) and Wilm’s tumor susceptibility gene 1 (WT1) immunostaining pattern. Engrafted OVA1X tumor also retained their levels of positive immunostaining for estrogen receptor through mouse-to-mouse passages. Ascitis or synchronous peritoneal implants arising through tumor perpetuation were rarely identified in mice (data not shown).

### Cisplatin treatment of engrafted tumor recapitulates characteristic features of primary tumor response in mice

OVA1X-implanted mice were treated with low (2 mg/kg), intermediate (3.5 mg/kg), and high (5 mg/kg) doses of cisplatin, and short- and long-term responses were evaluated (Fig. 1B). Low or intermediate doses of cisplatin were associated with a good short-term response, characterized by significant tumor weight reduction relative to the control group, whereas there was a complete response at high doses. Long-term response was investigated in a subgroup of mice (n = 4–6 mice/treatment/dose) that were kept alive for a postchemotherapy follow-up of 6 to 12 months. Tumors relapsed in 5 of 10 (50%) mice treated with 2 mg/kg and in 3 of 10 (30%) treated with 3.5 mg/kg at 6 months, whereas all animals treated with 5 mg/kg were disease-free after a 12-month follow-up. Postchemotherapy, histologic and immunohistochemical analysis of relapsed masses exhibited a viable serous adenocarcinoma that preserved the morphology and the main immunophenotypical characteristics of untreated engrafted tumors.

### In vivo development of a cisplatin-resistant engrafted tumor model that recapitulates cisplatin primary tumor behavior response is a feasible model for pharmacologic drug evaluations

The general approach used to obtain the cisplatin-resistant engrafted tumor model is illustrated in Fig. 1C. OVA1X-implanted mice were initially treated with low doses (2 mg/kg) of cisplatin. When tumors relapsed, they were harvested and implanted in new animals (mouse-to-mouse passage). The process was repeated up to 5 times by treating tumor-bearing mice with stepwise increasing doses of cisplatin (Fig. 1C). A progressively shortened time lag between treatment and tumor relapse was noted for the 3 independent tumor lines (named OVA1XR-L1, -L2, and -L3) generated after iterative cycles of treatment. Indeed, a shortened time lag was mainly noted after the third or fourth cycle, and became stabilized (41 ± 6.1 days) subsequently for successive cycles of cisplatin treatment (Fig. 1D, left). Next, we evaluated the levels of cisplatin tumor resistance by comparative assays of OVA1X and each of the 3 independent lines of resistant tumors and homogeneous resistance was reproduced with each individual OVA1XR tumors (Fig. 1D, right). Thus, we selected OVA1XR-L2 for all further experiments, hereafter referred to as OVA1XR. Figure 1A shows that OVA1X and OVA1XR both recapitulated the histologic and immunohistochemical patterns found in the original patient-derived tumor. Interestingly, a consistent loss of estrogen expression was observed among resistant OVA1XR tumor respect to primary and OVA1X.

Lurbinectedin is effective in the treatment of cisplatin-sensitive and cisplatin-resistant ovarian tumor models

OVA1X and OVA1XR were orthotopically implanted in mice and when homogeneous tumor sizes (300–500 mm³) were identified at palpation (on days 60 and 64 for OVA1X and OVA1XR, respectively) animals bearing tumors were randomized to the following groups (n = 8–12 mice/group): (i) placebo; (ii) cisplatin (3.5 mg/kg); (iii) lurbinectedin (0.180 mg/kg); and (iv) lurbinectedin + cisplatin (0.180 + 3.5 mg/kg). On day 21, cisplatin-sensitive tumors OVA1X experienced reductions of 95.3%, 88.3%, and 87.2% following the treatment with cisplatin, lurbinectedin, and lurbinectedin + cisplatin, respectively (Fig. 2A, left). Although, as single agents both cisplatin and lurbinectedin had a significant response with respect to the placebo-treated animals, nonsignificant differences were observed between both individual treatments. Likewise, combined lurbinectedin + cisplatin treatment had no additional significant benefit with respect to each individual treatment (lurbinectedin + cisplatin vs. cisplatin, P = 0.15; lurbinectedin + cisplatin vs. lurbinectedin, P = 0.85).

Figure 2B summarizes the results obtained for treatments of cisplatin-resistant tumor (OVA1XR), showing important differences between both tumors for lurbinectedin-based treatments. Thus, 48.2%, 93.6%, and 96.7% reductions in tumor weight were recorded following cisplatin, lurbinectedin, or lurbinectedin + cisplatin treatments, respectively. Lurbinectedin, as a single therapy was a significantly better response than with cisplatin (P = 0.003). Notably, the combined lurbinectedin + cisplatin treatment proved to be more active than either drug separately, suggesting a synergistic drug effect (lurbinectedin + cisplatin vs. lurbinectedin, P = 0.022; or vs. cisplatin, P = 0.002).

Histopathological changes were assessed for the different treatments within the tumor as surrounding stromal tissue in both cisplatin-sensitive (Fig. 2A, right, and Supplementary Table S1) and cisplatin-resistant tumors (Fig. 2B, right, and Table 1). Thus, enlargement of tumor cells, presence of
multinucleated giant cells, lymphocytic and histiocytic infiltrates with the presence of hemosiderin and fibrosis, and the scarring of tumoral stroma were observed associated with treatments. Interestingly, cisplatin treatment did not induce morphologic changes in the cisplatin-resistant OVA1XR tumors (Fig. 2B).

To investigate the long-term response a subgroup of treated mice (n = 4–6 mice/group) were kept alive post-chemotherapy. Thus, OVA1XR tumor relapse took place more than a period of 42 days in all cisplatin-treated mice, whereas in cisplatin-sensitive OVA1X regrowth was found in only 1 cisplatin-treated mouse after eight months follow-up. At sacrifice of OVA1XR, significant differences were found in the weight and histology of relapsed masses (RL) for lurbinectedin-based treatments compared with the cisplatin-RL group (lurbinectedin-RL vs. cisplatin-RL, P = 0.0020; lurbinectedin + cisplatin-RL vs. cisplatin-RL, P = 0.0008; Fig. 2C, top). Furthermore, combined lurbinectedin + cisplatin treatment was more active than lurbinectedin monotherapy (P = 0.046), suggesting a long-term synergistic antitumor response for combined therapy. This finding is reinforced by the histology of lurbinectedin + cisplatin-RL masses (Fig. 2C, bottom). Together, although our results showed the efficacy of lurbinectedin treatment in the treatment of cisplatin-sensitive and cisplatin-resistant orthotopic engrafted tumor models, it is of note that they also suggest a synergistic effect with cisplatin in cisplatin-resistant OVA1XR.
Table 1. Extensive histopathological tumor regression criteria analyses in postchemotherapy engrafted OVA1XR tumor

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<td>NHR</td>
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<td>0</td>
<td>0</td>
<td>1+</td>
<td>1+</td>
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<td>1+</td>
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<td>NHR</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>3+</td>
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<td>Lurbinectedin + cisplatin-RL</td>
<td>27</td>
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<td>NV</td>
<td>NV</td>
<td>NV</td>
<td>GHR</td>
<td></td>
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</tbody>
</table>

aFor short-term response studies, animals were sacrificed on day 21 of treatment, whereas for long-term-response studies, mice were sacrificed 42 days after the end of treatment when the tumor had relapsed.

bPattern and extent of tumor infiltration was classified as follows: 1+, macroscopic large confluent tumor mass(es); 2+, multiple small tumor foci; 3+, scattered solitary tumor cells or complete absence of residual tumor. The remaining regression criteria were graded as follows: 0/1+, no or only minimally presence of the regression criterion within the specimen; 2+, focal occurrence of the respective regression criterion; 3+, widespread occurrence of the respective regression criterion.

cCD, dystrophic calcification.

dNV, not evaluated by complete tumor regression. Characterized by the absence of macro- and microscopic lesions.

eThree histopathological response categories were defined based on the number of regression criteria: NHR, no histopathological response (≤1 regression criteria [3+] present); MHR, moderate (2 regression criteria [3+] present), and GHR, good histopathological response (≥3 regression criteria [3+] present).
Histopathological tumor regression criteria are associated with treatment response in cisplatin-sensitive OVA1X and cisplatin-resistant OVA1XR tumors

Cytotoxic therapy leads to morphologic and histopathological changes within tumor tissue as well in the involved stroma. Next, we evaluated histopathological tumor regression, which has been established as the gold standard for the assessment of treatment response in several types of solid tumors (42–45). Supplementary Table S1 and Table 1 show extensive analysis of regression criteria for both OVA1X and OVA1XR, to establish whether they are suitable indicators of treatment response, as described for primary solid tumors (42–45). Supplementary Table S1 and Table 1 were found for the combined treatment (Supplementary Table S1). Taken together, the tumor response and the histopathological regression criteria were evidence of the relevance of the combined treatment in cisplatin-sensitive OVA1X. In this context, in cisplatin-resistant OVA1XR tumor a good histopathological response was confirmed for the combined lurbinectedin + cisplatin treatment (Table 1). Moreover, the relevance of combined treatments was reinforced by the observation that the histopathological response was maintained in relapsed masses (Table 1).

Lurbinectedin and cisplatin treatments are synergistic in vivo in A2780-derived tumor xenografts

The synergism of the combined lurbinectedin + cisplatin treatment was further investigated in mice bearing A2780 xenografted tumors. Figure 3A shows the T/C values,
Lurbinectedin Inhibits Growth of Ovarian Cancer

Table 2. Dose-response treatment effect of s.c. xenografts of A2780-derived cell line

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose, mg/kg</th>
<th>Fraction affected&lt;sup&gt;a&lt;/sup&gt;, F&lt;sub&gt;a&lt;/sub&gt;</th>
<th>m (SD)</th>
<th>D&lt;sub&gt;m&lt;/sub&gt;</th>
<th>r</th>
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</thead>
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<td>0.29</td>
<td>1.04 (0.16)</td>
<td>2.12</td>
<td>0.978</td>
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<td></td>
<td>0.135</td>
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<td>0.090</td>
<td>0.22</td>
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<td></td>
<td>0.045</td>
<td>0.09</td>
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<tr>
<td>Cisplatin</td>
<td>6.0</td>
<td>0.45</td>
<td>1.24 (0.12)</td>
<td>1.29</td>
<td>0.990</td>
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<tr>
<td></td>
<td>4.5</td>
<td>0.31</td>
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<td>3.0</td>
<td>0.23</td>
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</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.12</td>
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<td></td>
</tr>
<tr>
<td>Lurbinectedin + cisplatin</td>
<td>0.180 + 6.0</td>
<td>0.77</td>
<td>2.34 (0.36)</td>
<td>1.20</td>
<td>0.977</td>
</tr>
<tr>
<td></td>
<td>0.135 + 4.5</td>
<td>0.56</td>
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<tr>
<td></td>
<td>0.090 + 3.0</td>
<td>0.50</td>
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<tr>
<td></td>
<td>0.045 + 1.5</td>
<td>0.10</td>
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</tbody>
</table>

<sup>a</sup>Fraction affected (F<sub>a</sub> = 1 – T/C), defined as the change in tumor volume for each treated (T) and placebo (C) groups during placebo-treated survival period.

<sup>b</sup>Derived from the median-effect plot: [log<sub>10</sub>F<sub>a</sub>/[1-F<sub>a</sub>]] versus log<sub>10</sub>(Dose), where m is the slope (as mean ± SD), D<sub>m</sub> is the intercept of the plot, and r is the linear regression coefficient.

defined as the change in tumor volume for each treated (T) and placebo (C) group during the placebo-treated survival period, for mice treated with lurbinectedin, cisplatin or combined lurbinectedin + cisplatin. Animals treated with high cisplatin doses showed the lowest T/C of 55.2% on day 10, whereas there was no antitumor effect induced by the lurbinectedin single-agent treatment (minimal T/C, 70.8% on day 4). The combined lurbinectedin + cisplatin treatment produced lower T/C values than the more active agent in this experiment (cisplatin at 6.0 mg/kg). The antitumor effect seen on day 4 (T/C, 39.8%) for the highest dose of the combination (0.180 + 6.0 mg/kg; lurbinectedin + cisplatin) increased on subsequent days (T/C, 23.4% on day 10). On day 10, lurbinectedin + cisplatin treatment displayed a dose-dependent antitumor effect with median tumor volumes (mm<sup>3</sup>) of 572.8, 1,074, 1,233, and 2,199 for animals treated with lurbinectedin + cisplatin at 0.180 + 6.0, 0.135 + 4.5, 0.09 + 3.0, and 0.045 + 1.5 mg/kg levels, respectively. Applying the median-effect principle to the data gave a Combination Index (CI) of 0.17 (at Fa = 0.97), suggesting a synergistic effect of the combination lurbinectedin + cisplatin in ovarian (A2780) xenografted tumors (Fig. 3B and Table 2).

Lurbinectedin-induced tumor response is mediated by antiproliferative and proapoptotic features and causes mitotic catastrophe

Next, we investigated whether tumor response mechanisms were induced by lurbinectedin associated with antiproliferative and proapoptotic features. Two experimental approaches were used: (i) in A2780-derived subcutaneous (s.c.) tumor xenografts treated with cisplatin, lurbinectedin or combined drugs for 24 or 72 hours; and (ii) in cisplatin-sensitive OVA1X and cisplatin-resistant OVA1XR tumors.

We found that 24 hours after treatments of the A2780 xenografts, the anti-phospho-Histone H3 (S10; H3S10ph) mitosis marker significantly decreased in cisplatin (P = 0.007), lurbinectedin (P = 0.002), or combined (P < 0.001) treatments compared with placebo-treated tumors (Fig. 4A). In fact, the decrease was significantly greater for the combined treatment than for each single therapy (lurbinectedin + cisplatin vs. lurbinectedin, P = 0.005; or vs. cisplatin, P = 0.015). In addition, a proapoptotic effect was associated with lurbinectedin treatments. Thus, a 6.7-fold increase in the number of apoptotic cells (by TUNEL assay) was observed in combined lurbinectedin + cisplatin (P = 0.013) treatment compared with the placebo group, and 3.0-fold and 3.7-fold increases with respect to cisplatin and lurbinectedin, respectively (Fig. 4C, left).

Likewise, antiproliferative and proapoptotic effects were confirmed in both engrafted orthotopic models. In OVA1XR (Fig. 4B), all treatments showed a significant decrease in the number of mitoses determined by H3S10ph (cisplatin, P = 0.007; lurbinectedin, P = 0.003; lurbinectedin + cisplatin, P < 0.001). As a single treatment, lurbinectedin was more effective than cisplatin (P = 0.044). Combined lurbinectedin + cisplatin treatment significantly diminished the number of mitoses with respect to single lurbinectedin (P = 0.016) or cisplatin (P = 0.003) treatment (Fig. 4B). This effect was also maintained in relapsed tumor masses (lurbinectedin + cisplatin-RL vs. cisplatin-RL, P = 0.005; or vs. lurbinectedin, P = 0.012). Apoptotic drug induction was assessed in OVA1X and OVA1XR by immunodetection in paraffin-embedded tissues of caspase-3, an early and specific apoptotic marker. In cisplatin-sensitive

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OVA1X, nonsignificant differences for the proapoptotic-induced effect were observed for the single treatments (cisplatin, 6.3-fold; lurbinectedin, 7.1-fold; \( P = 0.45 \); Fig. 4C, right). Whereas in cisplatin-resistant OVA1XR tumor the strong proapoptotic effect was noted for lurbinectedin (4.2-fold induction relative to the placebo, \( P = 0.014 \); and 2.8-fold with respect to cisplatin, \( P = 0.007 \)), cisplatin retaining a moderate capability of inducing apoptosis in OVA1XR tumors (1.5-fold induction relative to placebo, \( P = 0.036 \); Fig. 4C, right). We did not analyze apoptosis induction in the combined treatment because the extensive histopathological regression prevents the reliable interpretation of the caspase cleaved apoptosis assay (data not shown).

Finally, we investigated whether lurbinectedin treatments affected the morphology of the mitotic spindle by double immunofluorescence staining with \( \alpha \)-tubulin (red staining), a protein localized in the spindle, combined with staining with the mitosis marker histone H3S10ph (green staining; Fig. 4D and Supplementary Fig. 1). Thus, in mitotic cells identified by H3S10ph with vehicle-treated tumors, \( \alpha \)-tubulin shows that control cells display...
normal bipolar mitotic spindles with chromosomes correctly aligned on the metaphase plate. On the other hand, lurbinectedin-treated cells exhibited abnormal mitotic figures, with seriously defective chromosome alignment, and the cells displaying aberrant figures failed to progress through mitosis. The presence of cells displaying aberrant figures was particularly manifested for combined lurbinectedin + cisplatin treatment, in A2780-derived xenografts and both engrafted orthotopic models (Fig. 4D).

Discussion

In this work, we report the generation and characterization of a serous ovarian cancer model based on orthotopic tumor implantation in nude mice, and its further in vivo development as a tumor model of cisplatin resistance. Next, as preclinical models, we show that lurbinectedin, a new synthetic alkaloid binder to the DNA minor groove, is effective either in the treatment of cisplatin-sensitive and cisplatin-resistant ovarian tumors. So, our results show that the combination of 2 compounds that differentially bind the DNA major and minor grooves should be a useful treatment strategy for EOC patients, and suggest its importance for overcoming cisplatin resistance.

Recent data suggest an overall success rate of 10% for oncology products in clinical development, being one of the reasons attributed to this failure the fact that preclinical models used frequently do not predict clinical results (31). Currently, preclinical in vivo drug development is mainly realized in s.c. tumor xenografts generated after cell line injection, or in some cases after s.c. engraftment of primary tumor (29, 32, 33), and pure primary orthotopic tumor-based models have rarely been used. Few such tumor models are available: because surgery is often complex, small numbers of mice are used per study, and the models are more expensive (28). Here, we show that these orthotopic-based preclinical ovarian tumor models, which reproduce primary tumor properties, are outstanding resources for the development of new drug therapies. They would also be very valuable for exploring new therapeutic applications for drugs that are currently approved for use in humans, as we recently reported in microsatellite instability (MSI) + colorectal tumors with enoxacin (46). Thus, assessed chemotherapy responses in cisplatin-sensitive and cisplatin-resistant tumor models that maintain the morphologic, histologic, and genetic characteristics of patients’ tumors, including the behavior of the stromal component and the tissue architecture, may improve preclinical drug translation to patients.

To overcome cisplatin resistance and reduce the side effects, new agents should have different mechanisms of action and should be non–cross-resistant with platinum (47). Structurally, the DNA duplex gives rise to two well-defined clefts known as the major and minor grooves (25). While the DNA major groove represents a site of attack for cisplatin and many alkylating agents, other antitumor drugs such as eteicinascidins, mitomycin C and chromomycin A3 bind to the minor groove (16, 17). Our work with the new synthetic alkaloid lurbinectedin strongly suggests that strategies based on dual major and minor DNA groove-targeted therapies should be useful for treating cisplatin-resistant/refractory cases of ovarian carcinomas. Further studies in these models will allow a deeper insight into the cooperative mechanism of action among cisplatin and lurbinectedin, and enable their combined properties with other drugs such olaparib, temozolomide, doxorubicine, etc. to be evaluated. Although, lurbinectedin is structurally similar to trabectedin (Yondelis), their important different pharmacokinetics properties identified may lead to novel and/or increased antitumor activity compared with original trabectedin (25, 26).

Our preclinical findings in cisplatin-sensitive OVA1XR tumor indicate that lurbinectedin monotherapy treatment could be an active first-line drug on the basis of its similar cisplatin response rates and the related long-term behavior response. However, from the clinical standpoint, certainly, the most relevant preclinical result was the capability of lurbinectedin, either on its own or in combination with cisplatin, to overcome the cisplatin resistance of OVA1XR tumor. Its relevance was underlined by the better response and by the histopathological regression found in OVA1XR treated with lurbinectedin alone or combined with cisplatin, both in short- and long-term experiments. All together, our results indicate that combined lurbinectedin treatment should overcome cisplatin resistance, it being an effective second-line treatment for platinum responder patients as a first-line agent for refractory tumors.

In agreement with previously described in vitro results (25), we showed that the tumor response produced in vivo by lurbinectedin was mediated by an antiproliferative and proapoptotic induction and causes mitotic catastrophe. Previous reports showed common PM01183 (lurbinectedin) effectiveness in the nanomolar range in a panel of representative cell lines of different tumor types, and described that PM01183 (lurbinectedin) and cisplatin acted synergistically when tested in vitro on platinum-resistant cells lines (26). In this work, we showed that lurbinectedin synergizes in vivo with cisplatin treatments, an effect that is mainly observed in cisplatin-resistant OVA1XR tumors. It has been reported that although DNA lesions generated by lurbinectedin are not repaired by nucleotide excision repair (NER), it can interfere with NER, thereby attenuating the repair of specific NER substrates. Thus, lurbinectedin has enhanced activity in cisplatin-resistant cell lines with higher NER activity (26).

Dissemination in EOCs characteristically involves local invasion of pelvic and abdominal organs, and unlike many epithelial cancers, initial dissemination rarely requires the vascularure, although this is often involved in the advanced stages of disease (48). The tumor model presented here, as happens in their original patient, rarely disseminate in mice. Nevertheless, the generation of other orthotopic-based EOC tumors reproducing human dissemination patterns should be very useful for investigating drug action in malignant ascitis formation, the characteristic feature of advanced ovarian cancer at diagnosis. In fact, we have
developed other engrafted primary EOCs models that mimicked in mice human local and distal dissemination behaviors (A. Vidal and A. Villanueva, personal communication).

After neoadjuvant chemotherapy, the residual tumor size in ovarian specimens was the only histopathological criterion that was significantly associated with treatment and subsequent overall survival. Histopathological responders defined by the absence of residual tumor, scattered solitary tumor cells, or a residual tumor of 5 mm or less had a significantly longer survival (38). The size of the residual tumor remaining after debulking surgery is known to be an important prognostic factor (49). Likewise, we show in both tumor models that the size of the residual masses and the abundance of regressive criteria correlate with response. The strong correlation between our preclinical tumor models and clinical settings in terms of tumor progression and response to chemotherapy, strongly argues in favor of conducting clinical lurbinectin trials in resistant/refractory EOC.

In conclusion, we have shown that lurbinectin, a drug targeting the minor DNA groove, is active and in vivo synergizes with cisplatin, which targets the major DNA groove, in the treatment of orthotopic cisplatin-sensitive and cisplatin-resistant patient-derived preclinical tumor models. Overall, our results provide solid evidence supporting clinical trials with lurbinectin alone or in combination with cisplatin in advanced EOCs.

Disclosure of Potential Conflicts of Interest
M.J. Guillen, C. Cuevas, and P. Aviles are employees and shareholders of PharmaMar, SA (Madrid, Spain). No potential conflicts of interest were disclosed by the other authors.

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References
Lurbinectedin (PM01183), a New DNA Minor Groove Binder, Inhibits Growth of Orthotopic Primary Graft of Cisplatin-Resistant Epithelial Ovarian Cancer

August Vidal, Clara Muñoz, María-José Guillén, et al.


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