Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies

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Abstract
Histone deacetylase inhibitors represent a promising new class of anticancer agents. In the current investigation, we examined the activity of PXD101, a potent histone deacetylase inhibitor, used alone or in combination with clinically relevant chemotherapeutics (docetaxel, paclitaxel, and carboplatin), in preclinical in vitro and in vivo models of ovarian cancer. In vitro activity was examined in ovarian cancer and multidrug-resistant cell lines grown in monolayer culture, and in primary clinical ovarian cancer specimens grown in three-dimensional organoid culture. PXD101 was found to inhibit in vitro cancer cell growth at sub- to low micromolar IC50 potency, exhibited synergistic activity when used in combination with relevant chemotherapeutics, and effectively inhibited the growth of multidrug-resistant cells. In vivo, PXD101 displayed single-agent antitumor activity on human A2780 ovarian cancer s.c. xenografts which was enhanced via combination therapy with carboplatin. In support of these findings, PXD101 was shown to increase the acetylation of α-tubulin induced by docetaxel and the phosphorylation of H2AX induced by carboplatin. Taken together, these results support the clinical evaluation of PXD101 used alone or in combination therapy for the treatment of ovarian cancer. [Mol Cancer Ther 2006;5(8):2086–95]

Introduction
DNA alterations resulting from both genetic and epigenetic influences have been implicated in the development and maintenance of cancer (1–3). Genetic events such as DNA mutation, amplification, and chromosomal rearrangement may play roles in cancer by decreasing the expression or activity of tumor suppressor gene products and by increasing the expression or activity of proto-oncogene products. Epigenetic events, on the other hand, induce alterations in gene expression in the absence of DNA mutation and are believed to play a role in cancer primarily through the transcriptional repression of tumor suppressor genes (2, 4, 5). Although mechanistically distinct, both genetic and epigenetic events may target a partially overlapping subset of cancer-associated genes.

Significant advances have been made in the development of therapies to neutralize oncogetic proteins whose activities are inappropriately elevated in cancer due to overexpression or mutational activation attributable to genetic lesions. For example, tyrosine kinases have been successfully targeted therapeutically with small molecule inhibitors (e.g., Gleevec and Tarceva) and monoclonal antibodies (e.g., Herceptin and Erbitux; ref. 6). However, less success has been realized in the development of therapies that restore tumor suppressor gene expression or function in cancer cells following their loss due to genetic alteration. Although it may be possible to develop small molecules that restore tumor suppressor function (7), it remains a significant challenge to identify and therapeutically repair the genetic lesions which inactivate tumor suppressor genes within cancer cells.

In contrast, the restoration of expression of tumor suppressor genes that have been silenced by epigenetic events provides an attractive opportunity for therapeutic intervention. The two main epigenetic mechanisms of gene regulation that are exploited by cancer cells to mediate inappropriate gene expression are DNA methylation and histone modification. Hypermethylation of CpG-rich areas in the promoter regions of genes induces transcriptional silencing by blocking the access of transcription factors or by enhancing the binding of transcriptional repressors, and is believed to play an important role in cancer by causing a decrease in the expression of tumor suppressor genes (8). An inhibitor of DNA methylation, azacitidine, was recently approved by the Food and Drug Administration for the treatment of myeloplastic preleukemic syndromes (9). Aberrant histone modifications, such as hypoacetylation, have also been associated with malignancy through the transcriptional silencing of tumor suppressor genes (10, 11). Histones bind to DNA and regulate chromatin structure, and histone deacetylation mediates transcription repression by virtue of the fact that the removal of acetyl groups from histones allows them to interact more tightly with DNA, thereby limiting the accessibility of DNA for transcription. Histone acetylation is regulated by the opposing activities of histone acetyl transferase and histone deacetylase (HDAC) enzymes. Pharmacologic HDAC inhibitors from a number of chemical classes have shown promising anticancer activity in preclinical studies in which
they have been shown to induce growth arrest, differentiation, and apoptosis of cancer cells in vitro and anticancer activity in animal models. Although many of these compounds have been shown to enhance histone acetylation and to increase the expression of tumor suppressor genes in cancer cells, the precise mechanism(s) by which HDAC inhibitors inhibit tumor cell growth remains an area of active investigation and may involve the acetylation of both histone and nonhistone proteins. Some HDAC inhibitors evaluated in clinical trials have shown evidence of anticancer activity and low toxicity.

We have been studying a novel hydroxamate, PXD101, which inhibits the activity of a variety of HDACs with nanomolar IC50 potency and induces histone acetylation in tumor cells (12). This compound was previously shown to inhibit tumor cell growth in vitro and in an animal model (12), and is currently being evaluated as an anticancer agent in clinical trials. To support the clinical development of PXD101, preclinical studies were done to evaluate the activity of PXD101 used alone and in combination with various chemotherapeutics on a variety of cancer types.

With an estimated 22,220 new cases and 16,210 deaths in 2005 in the U.S. alone, ovarian cancer is the most lethal gynecologic neoplasm and ranks as the fourth leading cause of cancer-related deaths among American women (13–16). One contributing factor to the high mortality rate of this disease is that early stage ovarian cancer is frequently asymptomatic and difficult to detect, and thus many patients present with advanced disease, including i.p. metastases, at the time of diagnosis. Also, whereas many patients exhibit an initial response to chemotherapy, most will eventually succumb to drug-resistant metastases. Thus, ovarian cancer represents a significant unmet medical need.

Herein, we report our preclinical findings regarding the activity of PXD101 used as a single agent and in combination with clinically relevant chemotherapeutics (docetaxel, paclitaxel, and carboplatin). Our results support the clinical evaluation of PXD101 used alone or in combination therapy for the treatment of ovarian cancer.

Materials and Methods

Cell Lines

Parental and drug-resistant cell lines were obtained from the following sources: MES-SA, MES-SA/Dx5 (American Type Culture Collection, Manassas, VA); P388, P388/ADR (National Cancer Institute, Frederick, MD); KB-3-1, KB-V1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); NOMO-1, NOMO-1/ADM, K562, K562/ADM (Health Science Research Resources Bank, Osaka, Japan). OVCAR-3 and A2780 human ovarian cancer cell lines were obtained from the National Cancer Institute and the Piedmont Research Center (Wilmington, NC), respectively. Adherent cells were cultured in DMEM supplemented with 10% fetal bovine serum and suspension cells in RPMI 1640 supplemented with 10% fetal bovine serum.

In vitro Growth-Inhibition Assays on Ovarian Cancer Clinical Specimens

Testing was done by Oncotech (Tustin, CA) on cryopreserved chemotherapy-naïve ovarian cancer surgical specimens using the extreme drug resistance assay (17). In brief, specimens were plated as small organoid clusters in an agarose suspension, exposed to drug for 3 days and then pulsed with 3H-thymidine over an additional 2 days of culture. DNA was then harvested and the incorporation of 3H-thymidine into DNA was used as an indicator of cell growth. Following subtraction of background counts, the percentage of growth inhibition was calculated by dividing the average counts from drug-treated samples by that from control wells incubated in the absence of drug. Samples exhibiting <40% growth inhibition in the presence of 2 μmol/L paclitaxel and <45% growth inhibition in the presence of 6.7 μmol/L carboplatin were classified as drug-resistant, and samples exhibiting >40% growth inhibition in the presence of 2 μmol/L paclitaxel and >45% growth inhibition in the presence of 6.7 μmol/L carboplatin were classified as drug-sensitive. The 20 specimens used in the present study had been previously tested in the extreme drug resistance assay; 10 had been classified as resistant and 10 as sensitive to paclitaxel and carboplatin, and the present analysis is in good agreement with the classifications based on historical data. Pathologic examination indicated that 17 of the specimens are serous adenocarcinomas, 2 specimens (nos. 15 and 17) are serous adenocarcinomas.
tumors with low malignant potential that showed evidence of microinvasion, and 1 specimen is an endometrial adenocarcinoma (no. 4). Paclitaxel was obtained from Calbiochem (San Diego, CA) and was prepared as a 51.5 μmol/L stock in 5% DMSO/95% growth medium (RPMI 1640 supplemented with 15% fetal bovine serum and antibiotics). Carboplatin was obtained from Bristol Myers Squibb (New York, NY) and was prepared as a 215 μmol/L stock in PBS. PXD101 was prepared as a 20 mmol/L stock in DMSO.

Immunoblotting
Cells grown in monolayer culture were treated as indicated in the figure legends and harvested in 1× Tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA) supplemented with 10 mmol/L of DTT (Sigma). Cell lysates were boiled for 10 minutes, resolved on 4% to 20% gradient polyacrylamide gels (Invitrogen), and transferred to nitrocellulose filters (Invitrogen). Immunoblotting was done using standard procedures and the following primary antibodies: anti-acetylated (Lys40)-α-tubulin (Sigma), anti-phosphorylated (Ser139)-H2AX (Upstate, Charlottesville, VA), and anti-actin (Sigma). Following incubation with the appropriate horseradish peroxidase–conjugated secondary antibodies, enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, United Kingdom) was used for detection.

Animal Experiments
To initiate xenografts, female CD-1 athymic nude mice (Harlan Laboratories, Indianapolis, IN) were implanted s.c. with a 1 mm³ tumor fragment obtained from a human A2780 ovarian carcinoma xenograft. Once tumors became established (~100 mm³ in size), animals were randomized (10 animals/group) and drug treatments were initiated. Animals were 10 weeks of age when drug treatments were initiated. When administered, PXD101 was given by i.p. injection once daily for 15 consecutive days, whereas carboplatin was given by i.p. injection once daily for 5 consecutive days starting 7 days after the initiation of PXD101 treatment. Vehicle control animals were administered PXD101 vehicle (100 mg/mL L-arginine in water) by i.p. injection once daily for 15 consecutive days. Tumor measurements and animal body weights were recorded at least twice weekly. Tumor sizes were determined using the formula (width² × length) / 2. Tumor weight was estimated with the assumption that 1 mg is equivalent to 1 mm³ of tumor volume. Carboplatin was obtained from Sicor, Inc. (Irvine, CA) and was diluted in isotonic saline to 4 mg/mL for administration. PXD101 was prepared as a 50 mg/mL stock in PXD101 vehicle (pH ~9.4) and for administration was diluted in PXD101 vehicle to 10 mg/mL (for 100 mg/kg dose), 4 mg/mL (for 40 mg/kg

### Table 1. *In vitro* growth-inhibitory activity of PXD101 and docetaxel on multidrug resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>PXD101 (IC₅₀ μmol/L)</th>
<th>Docetaxel (IC₅₀ μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-3-1</td>
<td>Cervical</td>
<td>0.446</td>
<td>0.003</td>
</tr>
<tr>
<td>KB-V1</td>
<td>Drug-resistant variant</td>
<td>1.07</td>
<td>0.513</td>
</tr>
<tr>
<td>Fold resistance</td>
<td></td>
<td>2.4</td>
<td>171</td>
</tr>
<tr>
<td>MES-SA</td>
<td>Uterine</td>
<td>4.12</td>
<td>0.051</td>
</tr>
<tr>
<td>MES-SA/Dx5</td>
<td>Drug-resistant variant</td>
<td>3.78</td>
<td>7.820</td>
</tr>
<tr>
<td>Fold resistance</td>
<td></td>
<td>0.92</td>
<td>153</td>
</tr>
<tr>
<td>NOMO-1</td>
<td>Leukemia</td>
<td>0.548</td>
<td>0.002</td>
</tr>
<tr>
<td>NOMO-1/ADM</td>
<td>Drug-resistant variant</td>
<td>0.655</td>
<td>0.472</td>
</tr>
<tr>
<td>Fold resistance</td>
<td></td>
<td>1.2</td>
<td>236</td>
</tr>
<tr>
<td>P388</td>
<td>Leukemia</td>
<td>0.183</td>
<td>0.003</td>
</tr>
<tr>
<td>P388/ADR</td>
<td>Drug-resistant variant</td>
<td>0.345</td>
<td>3.340</td>
</tr>
<tr>
<td>Fold resistance</td>
<td></td>
<td>1.9</td>
<td>1113</td>
</tr>
<tr>
<td>K562</td>
<td>Leukemia</td>
<td>1.12</td>
<td>0.064</td>
</tr>
<tr>
<td>K562/ADM</td>
<td>Drug-resistant variant</td>
<td>2.03</td>
<td>0.894</td>
</tr>
<tr>
<td>Fold resistance</td>
<td></td>
<td>1.8</td>
<td>14</td>
</tr>
</tbody>
</table>

NOTE: All cell lines except P388 and P388/ADR are of human origin. Cells were grown in monolayer culture (KB-3-1, KB-V1, MES-SA, and MES-SA/Dx5) or in suspension (NOMO-1, NOMO-1/ADM, P388, P388/ADR, K562, and K562/ADM), and IC₅₀ values were extrapolated from growth curves obtained using the CellTiter-Glo assay following 72 hours of treatment with nine different concentrations of drug. Fold resistance was calculated by dividing the IC₅₀ of the drug-resistant variant cell line by that of the corresponding parental cell line.
Table 2. In vitro growth-inhibitory activity of PXD101, paclitaxel, and carboplatin on ovarian cancer clinical specimens

<table>
<thead>
<tr>
<th>Drug treatment (μM/L)</th>
<th>Sample type</th>
<th>Sensitive</th>
<th>Resistant</th>
<th>Sensitive + resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>PXD101 (2.5)</td>
<td>86</td>
<td>81</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.63)</td>
<td>68</td>
<td>38</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.16)</td>
<td>44</td>
<td>13</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel (2.0)</td>
<td>59</td>
<td>20</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel (0.02)</td>
<td>50</td>
<td>14</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Carboplatin (6.7)</td>
<td>60</td>
<td>26</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Carboplatin (0.74)</td>
<td>20</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.16)/Paclitaxel (2.0)</td>
<td>75</td>
<td>28</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.16)/Paclitaxel (0.02)</td>
<td>68</td>
<td>31</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.16)/Carboplatin (6.7)</td>
<td>77</td>
<td>38</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>PXD101 (0.16)/Carboplatin (0.74)</td>
<td>54</td>
<td>16</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Ovarian cancer clinical specimens which are considered relatively sensitive (n = 10) or resistant (n = 10) to paclitaxel and carboplatin were grown in three-dimensional organoid culture and assessed for drug sensitivity. Sensitive + resistant refers to the average obtained for all samples examined (n = 20). Results are reported as the percentage of growth inhibition relative to control samples cultured in the absence of drug, and were obtained using a thymidine-incorporation assay following 5 days of drug treatment.
also tended to be less readily growth-inhibited by PXD101 (mean growth inhibitory rates of 68% and 38%; \( P = 0.05 \) for PXD101 used at 0.63 \( \mu \)mol/L and compared on paclitaxel/carboplatin-sensitive and -resistant samples, respectively; mean growth inhibitory rates of 44% and 13%; \( P = 0.007 \) for PXD101 used at 0.16 \( \mu \)mol/L and compared on paclitaxel/carboplatin-sensitive and resistant samples, respectively).

**In vitro Growth-Inhibitory Activity of PXD101 Used in Combination with Other Chemotherapeutics on Ovarian Cancer Cell Lines and Clinical Specimens**

Because chemotherapy for ovarian cancer often involves combination therapy with drugs such as docetaxel, paclitaxel, and carboplatin, we determined the activity of PXD101 used in combination with these compounds. Carboplatin binds to and cross-links DNA, thereby triggering apoptosis or necrosis if the DNA cannot be properly repaired in a timely fashion (18). Docetaxel and paclitaxel bind to and stabilize tubulin, which suppresses microtubule dynamics, thereby inhibiting mitosis and stimulating apoptosis (19). Because docetaxel and paclitaxel have been shown to work equally well for ovarian cancer (20), we investigated both of these compounds.

Ovarian cancer cell lines grown in monolayer culture were treated with PXD101 in the presence or absence of docetaxel or carboplatin for 48 hours and then assessed for viability/growth via the CellTiter-Glo assay. Data are plotted as the percentage of growth inhibition relative to untreated cells. *, drug combinations generating a statistically significant enhancement of growth-inhibitory activity compared with either drug used as a single agent. Columns, means from a single experiment done in triplicate; bars, SD. The experiments were repeated with similar results. PXD101 (\( \text{PXD} \)) and carboplatin (\( \text{CP} \)) concentrations are in micromoles, and docetaxel (\( \text{DT} \)) is in nanomoles. Below each graph are drug combination results analyzed for potential synergy by CalcuSyn software which were determined following treatment of cells with various concentrations of PXD101 and docetaxel or carboplatin in a matrix format (see Materials and Methods).
interactions shown here for cell lines grown as monolayers and analyzed by the CellTiter-Glo assay were also evident in a separate set of experiments in which an ovarian cancer cell line was cultured in three-dimensional soft agar and analyzed by a clonogenic assay (see Supplemental Table S3).4

The activity of PXD101 (0.16 μmol/L) used in combination with paclitaxel or carboplatin on ovarian cancer clinical specimens grown in three-dimensional organoid culture and analyzed by thymidine incorporation was also examined (Table 2; Supplemental Table S4).4 This analysis shows that PXD101 used in combination with either paclitaxel or carboplatin generally leads to enhanced growth inhibitory activity compared with the corresponding drugs used as monotherapies. For example, PXD101 (0.16 μmol/L) and paclitaxel (0.02 μmol/L) generated growth inhibition means of 29% and 32%, respectively, whereas the drug combination generated a growth inhibition mean of 49%. This represents significantly greater activity by the drug combination compared with PXD101 used as a single agent (P = 0.021) or paclitaxel used as a single agent (P = 0.038).

**Tumor Growth-Inhibitory Activity of PXD101 and Drug Combinations in a Human Ovarian Cancer Xenograft Model**

The activity of PXD101 used as a single agent or in combination with carboplatin on human A2780 ovarian cancer xenografts was examined (Fig. 2). Athymic nude mice harboring established s.c. xenografts were treated i.p. with PXD101 monotherapy at three different concentrations (20, 40, or 100 mg/kg per dose), carboplatin monotherapy at a single concentration (40 mg/kg/dose), or combinations of both PXD101 and carboplatin. In clinical trials, chemotherapeutics administered i.p. to patients with ovarian cancer have shown a survival benefit compared with the same drugs given i.v. (21).

**Figure 2.** *In vivo* antitumor activity of PXD101 used alone or in combination with carboplatin on human ovarian cancer xenografts. Athymic nude mice harboring small established s.c. xenografts of human A2780 ovarian cancer cells were used to evaluate potential antitumor activity. Results are plotted as the mean tumor volumes (mm³) that were obtained by measuring the tumors at the indicated time points. The activity of PXD101 (PXD) used at 20, 40, or 100 mg/kg/d alone or in combination with carboplatin (CP) is presented in (A), (B), and (C), respectively. When administered, PXD101 was given once daily for 15 d beginning on day 1, a time at which tumor volumes had attained a size of ~100 mm³, whereas carboplatin was given every other day beginning on day 7 and was continued for a total of five doses. Both PXD101 and carboplatin were administered via i.p. injection. The initial number of animals/group was 10. By the time of the final tumor measurement (i.e., day 15), one animal had been lost from the vehicle control group and the group coadministered carboplatin/PXD101 (20 mg/kg), and the means from these groups at this time point were derived from the remaining nine animals. Drug concentrations (mg/kg/injection) are shown in parentheses. The percentage of decrease in mean tumor volume in drug-treated relative to vehicle-treated animals at day 15 is indicated on the graphs; *, statistically significant tumor growth inhibition relative to vehicle-treated animals. **D**, the mean number of days for the tumors in each treatment group to reach 2,000 mm³; *, statistically significant tumor growth inhibition relative to vehicle-treated animals.
Results indicate that PXD101 monotherapy induced dose-proportional antitumor effects. When administered at 100 mg/kg (Fig. 2C), PXD101 inhibited tumor size by 47% ($P = 0.012$) at day 15 relative to vehicle-treated control animals. PXD101 administered at 20 and 40 mg/kg (Fig. 2A and B) also decreased tumor size relative to the vehicle control group, but these effects did not reach statistical significance (27% for 20 mg/kg, $P = 0.142$; and 34% for 40 mg/kg, $P = 0.074$).

When PXD101 was administered in combination with carboplatin, the antitumor effects were greater than those of either compound given as monotherapy (Fig. 2A–C). For example, PXD101 (20 mg/kg) administered in combination with carboplatin significantly inhibited tumor size (51%, $P = 0.011$) at day 15 relative to vehicle-treated animals, whereas the relevant monotherapies did not (19%, $P = 0.331$ for carboplatin; and 27%, $P = 0.142$ for PXD101; Fig. 2A). Moreover, a comparison of the PXD101 (20 mg/kg) / carboplatin combination group with the relevant monotherapy groups indicates that the combination induced significantly greater tumor growth inhibition compared with either PXD101 or carboplatin monotherapy ($P = 0.041$ and $P = 0.029$, respectively).

As an additional measure of antitumor activity, the mean number of days that it took for the tumors in each group to reach a volume of 2,000 mm$^3$ was extrapolated (Fig. 2D). The results of this analysis indicate that PXD101 monotherapy (100 mg/kg) significantly delayed tumor growth relative to vehicle-treated control animals (mean of 14.6 days for the vehicle-treated group compared with 18.6 days for the PXD101-treated group; $P = 0.048$). Importantly, the combination of PXD101 (100 mg/kg) with carboplatin (40 mg/kg) delayed tumor growth even further (mean of 22.5 days), despite the fact that carboplatin monotherapy (mean of 15.0 days) did not significantly delay tumor growth relative to vehicle-treated animals.

In the xenograft experiment described above, none of the drug treatments caused weight loss of >10% or overt toxicity other than local injection site ulceration.

### Effect of PXD101 and Docetaxel on the Acetylation of $\alpha$-Tubulin

Docetaxel and paclitaxel bind to and stabilize tubulin (19). The acetylation of tubulin is believed to be an indicator of tubulin stability (22), and tubulin-stabilizing compounds have been shown to enhance tubulin acetylation. Some HDAC inhibitors have also been shown to stimulate tubulin acetylation, an effect that is likely mediated via the inhibition of HDAC6 (23). To investigate the possibility that PXD101 enhances tubulin acetylation, lysates from ovarian cancer cells that had been treated with various doses of PXD101 for 24 hours were immunoblotted with an antibody to acetylated tubulin (Fig. 3A). This analysis shows that both PXD101 and docetaxel each enhance tubulin acetylation in a dose-dependent fashion, and that cells treated with a combination of these compounds exhibit enhanced tubulin acetylation compared with cells treated with either drug used as monotherapy.

### Effect of PXD101 and Carboplatin on the Phosphorylation of Histone H2AX

Platinum agents such as cisplatin and carboplatin bind to and cross-link DNA, leading to DNA damage (18). An early cellular response to DNA damage is the phosphorylation of the histone H2AX, an event which leads to chromatin remodeling that is designed to facilitate DNA repair (24, 25). Because H2AX phosphorylation may reflect the level of DNA damage and subsequent repair following the treatment of cells with platinum agents, we examined the extent of this modification following the treatment of ovarian cancer cells for 24 hours with PXD101 or carboplatin used alone or in combination (Fig. 3B). This analysis shows that PXD101 does not by itself induce H2AX phosphorylation, whereas carboplatin does. In addition, the combination of PXD101 and carboplatin induced a greater level of H2AX phosphorylation than did either compound used as monotherapy.

### Discussion

In the present preclinical investigation, we found that PXD101 monotherapy inhibits the in vitro growth of...
human ovarian cancer cell lines grown in monolayer culture and ovarian cancer clinical specimens grown in three-dimensional organoid culture at low to submicromolar IC₅₀ concentration. When examined in vivo, PXD101 monotherapy delayed the growth of human ovarian cancer xenografts. Our finding that an HDAC inhibitor is active as a stand-alone agent on ovarian cancer in preclinical studies is consistent with published reports (12, 26–29). Importantly, our experiments also show that PXD101 works in an additive to synergistic manner, both in vitro and in animal models, when used in combination with chemotherapeutics that are clinically relevant for the treatment of ovarian cancer, and that PXD101 is active on drug-resistant cancer cell lines and ovarian cancer clinical specimens.

The potent growth-inhibitory activity of PXD101 that was evident on ovarian cancer cell lines grown in monolayer culture was also found when this compound was examined on primary ovarian cancer clinical specimens grown in three-dimensional organoid cultures. Three-dimensional organoid culture is thought to more closely mimic the in vivo microenvironment than does monolayer culture, and cells grown in this fashion are often more resistant to the growth-inhibitory effects of chemotherapeutic agents than are cells grown in monolayer culture. This finding may reflect the slower growth rate of three-dimensional organoids and the inability of some drugs to adequately penetrate multicellular organoid structures (30). Likewise, primary tumor cultures are believed to be more representative of in vivo tumors than are established cancer cell lines and generally display weaker responses to chemotherapeutics than do cell lines (31). Thus, it is notable that PXD101 displays strong growth-inhibitory activity on primary ovarian cancer clinical specimens grown in three-dimensional culture.

The topic of drug resistance is particularly relevant to ovarian cancer because many such patients initially respond to chemotherapy, but eventually succumb to drug-resistant metastases (14–16). In the present investigation, we examined the activity of PXD101 in relation to drug resistance in two ways. First, using parental and drug-resistant cell line variants in monolayer culture, we found that the growth-inhibitory activity of PXD101 was not strongly influenced by the drug-resistant status of the cells. Most of the drug-resistant cell lines used in the present investigation reportedly overexpress P-glycoprotein, and similar results were obtained from cells that reportedly overexpress MRP (data not shown). Second, using ovarian cancer clinical specimens grown in three-dimensional culture, we found that PXD101 (2.5 μmol/L) exhibited strong growth-inhibitory activity on ovarian cancer clinical specimens that were relatively resistant to paclitaxel and carboplatin, two drugs that are commonly used to treat ovarian cancer, although PXD101 used at lower doses (0.63 and 0.16 μmol/L) in this analysis was influenced by drug resistance status. In a previous study, PXD101 showed a moderate level of cross-resistance on a cisplatin-resistant ovarian cancer cell line in vitro and inhibited the tumorigenic growth of this cell line in vivo (12). The combined data suggests that the activity of PXD101 is not strongly influenced by common mechanisms (e.g., overexpression of P-glycoprotein or MRP) that may mediate resistance to other drugs, including drugs that are used for the treatment of ovarian cancer, and suggests that PXD101 may be active on ovarian cancers that are resistant to standard-of-care chemotherapeutics. A recent study showed that some HDAC inhibitors were cytostatic on parental ovarian cancer cells, but were cytotoxic on cisplatin-resistant ovarian cancer cells, thus raising the possibility that some HDAC inhibitors may actually work better on tumors that have developed resistance to other chemotherapeutics (28). However, other HDAC inhibitors, of a different chemical class than PXD101, seem to be strongly influenced in a negative fashion by P-glycoprotein and MRP (32, 33). Whether PXD101 or other structurally similar HDAC inhibitors will show a clinical advantage over HDAC inhibitors that are reportedly substrates for P-glycoprotein/MRP remains to be determined.

Drug combinations are sometimes used to treat cancer and the simultaneous use of multiple anticancer drugs, especially those that do not exhibit cross-resistance, is a conceptually attractive idea. The heterogeneous nature of most tumors makes it likely that different cells within the tumor will exhibit differential drug responses, and thus the simultaneous administration of multiple drugs may target a greater percentage of tumor cells than just a single drug. Moreover, some drugs work in a synergistic fashion such that the presence of one drug enhances the effectiveness of the other. However, potential benefits of combination therapy must be balanced against the potential for increased toxicity or antagonistic drug interactions. The in vitro findings in the present report, which show that PXD101 is synergistic when used in combination with chemotherapeutics relevant for the treatment of ovarian cancer, are in general agreement with results that have been obtained by other groups using HDAC inhibitors other than PXD101 (27, 34–38). The in vivo findings in the present report show that PXD101 used in combination with carboplatin in animals harboring A2780 ovarian cancer xenografts mediates greater antitumor activity than either drug used as monotherapy, apparently without an increase in toxicity. Similar results were found when PXD101 was used in combination with carboplatin or paclitaxel in an animal survival model in which P388 murine leukemia cells were injected into the i.p. cavity of mice (data not shown). The dose of carboplatin administered to mice in the present investigation (40 mg/kg; equivalent to ~ 120 mg/m²) was selected, based on prior experience, as a dose which was expected to exhibit only partial tumor growth inhibition so that the potential synergy with PXD101 could be examined. The concentration of carboplatin administered to humans in the clinic is 360 mg/m². The doses of PXD101 administered to mice in the present investigation were 20 to 100 mg/kg (equivalent to ~ 60–300 mg/m²), whereas the dose of PXD101 that has been determined to be generally safe in humans and is being
used in phase II clinical trials is 1,000 mg/m². The appropriate drug concentrations for human use should be determined in a clinical setting because mice do not reliably predict drug concentrations that are most appropriate for use in humans.

The mechanism(s) by which PXD101, or other HDAC inhibitors, synergize with paclitaxel or docetaxel is poorly understood. Interestingly, synergy among different tubulin-targeting drugs has been previously described (19, 38). We have shown that both PXD101 and docetaxel monotherapies mediate tubulin hyperacetylation, which is a hallmark of tubulin stabilization (22), and that the combination of these two compounds leads to an even greater level of tubulin acetylation than is seen with the respective monotherapies. Thus, tubulin is a common target of both PXD101 and docetaxel. It is likely that PXD101 mediates tubulin hyperacetylation via the inhibition of HDAC6, which is a known tubulin deacetylase (23). Consistent with this possibility, we have found that PXD101 potently inhibits the enzymatic activity of purified recombinant HDAC6 (IC₅₀ of 0.082 μmol/L).³ The finding that tubacin, which is reportedly an HDAC6-specific inhibitor that increases the acetylation of tubulin but not histones (39), enhances the activity of paclitaxel (38), suggests that the inhibition of HDAC6 by PXD101 may contribute to the synergistic effects that are observed when PXD101 is used in combination with docetaxel or paclitaxel. In addition, the finding that trichostatin A which, like PXD101, inhibits a variety of HDACs including HDAC6, and enhances the acetylation of tubulin, influences microtubule stability (40), suggests that the enhanced acetylation of tubulin by PXD101 may have functional consequences. However, our data is only suggestive and not direct proof of a functional relationship between the hyperacetylation of α-tubulin by both PXD101 and docetaxel and the synergistic growth-inhibitory activity shown by this drug combination.

Likewise, it is unclear how PXD101 or other HDAC inhibitors synergize with carboplatin. Our observation that PXD101 used in combination with carboplatin mediates the phosphorylation of histone H2AX to a greater degree than does either of these compounds administered as monotherapies suggests that PXD101 may increase the level of DNA damage induced by carboplatin. Other HDAC inhibitors have been found to increase the cytotoxic activity of anticancer drugs that target DNA, possibly by enhancing their access to DNA following HDAC inhibitor–mediated chromatin relaxation (34), and our results are consistent with this hypothesis.

In summary, these preclinical results using a variety of in vitro and in vivo cancer models show that PXD101 monotherapy is active on ovarian cancer and that PXD101 can be effectively combined with relevant chemotherapeutics to afford an even greater level of activity than is achieved with the respective monotherapies. These findings support the clinical evaluation of PXD101 used alone or in combination therapy for the treatment of ovarian cancer, including cancers that may exhibit resistance to commonly used chemotherapeutics.

References


Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies

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