Small-Molecule RA-9 Inhibits Proteasome-Associated DUBs and Ovarian Cancer In Vitro and In Vivo via Exacerbating Unfolded Protein Responses

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Abstract

Purpose: Ovarian cancer is the deadliest of the gynecologic malignancies. Carcinogenic progression is accompanied by upregulation of ubiquitin-dependent protein degradation machinery as a mechanism to compensate with elevated endogenous proteotoxic stress. Recent studies support the notion that deubiquitinating enzymes (DUB) are essential factors in proteolytic degradation and that their aberrant activity is linked to cancer progression and chemoresistance. Thus, DUBs are an attractive therapeutic target for ovarian cancer.

Experimental Design: The potency and selectivity of RA-9 inhibitor for proteasome-associated DUBs was determined in ovarian cancer cell lines and primary cells. The anticancer activity of RA-9 and its mechanism of action were evaluated in multiple cancer cell lines in vitro and in vivo in immunodeficient mice bearing an intraperitoneal ES-2 xenograft model of human ovarian cancer.

Results: Here, we report the characterization of RA-9 as a small-molecule inhibitor of proteasome-associated DUBs. Treatment with RA-9 selectively induces onset of apoptosis in ovarian cancer cell lines and primary cultures derived from donors. Loss of cell viability following RA-9 exposure is associated with an unfolded protein response as mechanism to compensate for unsustainable levels of proteotoxic stress. In vivo treatment with RA-9 retards tumor growth, increases overall survival, and was well tolerated by the host.

Conclusions: Our preclinical studies support further evaluation of RA-9 as an ovarian cancer therapeutic.

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Introduction

The ubiquitin–proteasome system (UPS) is responsible for >80% of the intracellular protein degradation in eukaryotes and consists of three components: the proteasomes, the ubiquitin-conjugating system, and the deubiquitinating enzymes (DUB; ref. 1). Although the ubiquitin-conjugating system is responsible for initiating the cascade leading to protein degradation, DUBs deconjugate ubiquitin from targeted proteins. This step is essential for protein degradation as deubiquitination is required for regulating proteasome-mediated protein degradation. The human genome encodes for approximately 100 DUBs that can be grouped based on their catalytic domain into two families: cysteine proteases and metalloproteases. Within the cysteine-based DUB family, ubiquitin-specific proteases (USP) and ubiquitin C-terminal hydrolases (UCH) are the most represented members constituting >90% of the mammalian cell DUBs pool (2, 3). Importantly, members of both USP and UCH families are differentially expressed and activated in a number of cancer types and their aberrant activity linked to cancer progression, and the onset of chemoresistance. Thus, DUBs have been suggested as a potential therapeutic targets for cancer treatment (4–9).

Ovarian cancer is a heterogeneous disease with respect to histopathology and molecular biology (10), yet it is accompanied by progressive cellular adaptation to cope with increasing levels of metabolic stress inherent to the cancer phenotype (i.e., rapid proliferation, elevated oxidative and proteotoxic stress; refs. 11–14). Previously, we showed that ovarian cancer cells are under higher levels of ubiquitin–proteasome stress as compared with normal cells, rendering them selectively sensitive to inhibition of...
Ovarian cancer is the fifth leading cause of cancer deaths among American women. Ovarian cancer is associated with upregulation of protein degradation machinery presumably as a mechanism to sustain the metabolic demands inherent to the malignant phenotype of ovarian cancer cells. In proteasome-mediated proteolysis, deubiquitinating enzymes (DUB) are an essential component of the ubiquitin-dependent protein degradation pathway. Owing to their recently described roles during cancer progression and chemoresistance, deubiquitinating enzymes are an attractive new target for ovarian cancer treatment. Here, we report the characterization of RA-9 as a small-molecule inhibitor of proteasome-associated DUB activity. We show that treatment with RA-9 selectively kills ovarian cancer cells, including primary cells derived from patient tumors. Mechanistically, RA-9 exposure induces onset of apoptosis via an unresolved unfolded protein response, including activation of endoplasmic reticulum stress responses. Our preclinical studies using immunodeficient mice bearing an intraperitoneal xenograft model of human ovarian cancer shows that treatment with RA-9 retards tumor growth and increases overall survival with no apparent toxicity on the host. Taken together, our preclinical studies support further evaluation of this small-molecule inhibitor of proteasome-associated DUBs alone, or in combination with conventional or other novel therapies, for ovarian cancer treatment.

Translational Relevance
Ovarian cancer is the fifth leading cause of cancer deaths among American women. Ovarian cancer is associated with upregulation of protein degradation machinery presumably as a mechanism to sustain the metabolic demands inherent to the malignant phenotype of ovarian cancer cells. In proteasome-mediated proteolysis, deubiquitinating enzymes (DUB) are an essential component of the ubiquitin-dependent protein degradation pathway. Owing to their recently described role during cancer progression and chemoresistance, deubiquitinating enzymes are an attractive new target for ovarian cancer treatment. Here, we report the characterization of RA-9 as a small-molecule inhibitor of proteasome-associated DUB activity. We show that treatment with RA-9 selectively kills ovarian cancer cells, including primary cells derived from patient tumors. Mechanistically, RA-9 exposure induces onset of apoptosis via an unresolved unfolded protein response, including activation of endoplasmic reticulum stress responses. Our preclinical studies using immunodeficient mice bearing an intraperitoneal xenograft model of human ovarian cancer shows that treatment with RA-9 retards tumor growth and increases overall survival with no apparent toxicity on the host. Taken together, our preclinical studies support further evaluation of this small-molecule inhibitor of proteasome-associated DUBs alone, or in combination with conventional or other novel therapies, for ovarian cancer treatment.

Materials and Methods
Reagents and plasmids
The deubiquitinating enzyme inhibitor RA-9 was synthesized and purified as we have previously described (15). The proteasome inhibitor bortezomib was purchased from Cayman Chemical. Cisplatin (cis-Diaminodichloroplatinum(II)) was purchased from Sigma-Aldrich. The 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (WST-1) was purchased from Roche Diagnostics GmbH. The CellTiter96 AQueous One Solution Cell Proliferation Kit was purchased from Promega. The 19S RP (Cat. # E-366) and the Ub-AMC substrate (Cat. # U-550) were purchased from BioVision. The lentivirus vector pEF-GFP-SIN, the VSVG envelope, and the delta-8.9 plasmids were kindly provided by Dr. Koho Iizuka (University of Minnesota, MN).

Cell lines and transfection
Human ovarian cancer cell lines SKOV-3, TOV-21G, OVCAR-3, and ES-2 were obtained from ATCC and cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 5% CO2. The ovarian cancer cell line HEY was a generous gift from Dr. Sundaram Ramakrishnan (University of Minnesota, MN) and was cultured as described above. The MM cell lines RPMI8226 and ANBL6 and their bortezomib-resistant clones RPMI8226-V10R and ANBL6-V10R were provided by Dr. Robert Z. Orlowski (M.D. Anderson Cancer Center, TX) and were cultured as previously described (16). For in vivo experiments, subconfluent cultures of ES-2 ovarian cancer cells were infected with lentiviral particles expressing the GFP reporter as we have previously described (17, 18).

Ub-AMC protease assay on 19S RP
Residual 19S RP was measured on purified 19S RP as previously described (18). Briefly, 19S RP (5 nmol/L) was incubated in DUB buffer (20 mmol/L Hepes, 0.5 mmol/L EDTA, 5 mmol/L DTT, and 0.1 mg/mL BSA, pH 7.8) with the indicated concentration of drugs in a 100-μL reaction volume for 60 minutes at room temperature, and the reaction was initiated by the addition of 500 nmol/L of the fluorogenic substrate Ub-AMC. Release of the AMC fluorophore was recorded using a plate-reading luminometer equipped with 380-nm excitation and 440-nm emission filters (Molecular Devices). All experiments were performed in triplicate.

Ub-AMC protease assay on whole cell lysate
To measure the inhibition of deubiquitinating enzyme activity on whole cell lysate, exponentially growing ES-2 cells were incubated with the indicated drug concentrations for 18 hours. Cells were lysed in DUB lysis buffer (25 mmol/L Hepes, 5 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L ATP), the nuclei were removed by centrifugation, and 100-μL of supernatant was incubated with equal volume of Ub-AMC (500 nmol/L) at room temperature for 30 minutes. Release
Antibodies and Western blot analysis

All experiments were performed in triplicate.

**Cell viability assay**

Cell viability was determined by WST-1 or CellTiter96 AQueous One Solution Cell Proliferation assays as previously described (15–17). Briefly, cells were seeded at the concentration of 1,000 or 10,000 per well in 100-μL medium in 96-well plate and treated with the indicated concentrations of drugs. At the indicated time points, cells were incubated according to the manufacturer’s protocol with the WST-1 or CellTiter96 labeling mixture. Formazan dye was quantified using a spectrophotometric plate (ELISA reader 190; Molecular Devices). All experiments were performed in triplicate.

**Antibodies and Western blot analysis**

Total cellular protein (10–20 μg) from each sample was separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to Western blot analysis. Antibodies for Western blot analysis were obtained from the following commercial sources: anti-ubiquitin (Santa Cruz Biotechnology and Millipore), anti-PCNA (Abcam), anti-PARP (BD Pharmingen), anti-GRP78, glutinin (Santa Cruz Biotechnology and Millipore), anti-ubiquitin, and anti-IRE1α, anti-Ero1L-α, and anti-caspase-3 (Cell Signaling), anti-β-actin (Sigma). Peroxidase-linked anti-mouse Immunoglobulin G and peroxidase-linked anti-rabbit Immunoglobulin G were from Amersham.

**Flow cytometry**

Cell cycle status was analyzed with a FACSCompur flow cytometer (Becton Dickinson) by measuring fluorescence from cells stained with propidium iodide (PI; Sigma) following drug treatment. For active caspase-3 experiments, cells were treated for the indicated amount of time, harvested, and immediately stained with the FITC-conjugated antiactive caspase-3 antibody according to the protocol provided by the manufacturer.

**Animals**

Six-week-old female immunodeficient (NCr nu/nu) mice were obtained from National Cancer Institute-Frederick (Frederick, MD) and maintained in a pathogen-free animal facility at least 1 week before use. All animal studies were done in accordance with institutional guidelines following approval by the IACUC.

**Xenograft murine model**

Mice were inoculated i.p. with 100,000 ES-2 cells (in 100 μL DMEM) stably expressing GFP. When tumor was detectable (approximately 6 days after inoculation), mice were randomly assigned into two groups receiving RA-9 or 0.9% saline. Treatment with RA-9 was given i.p. on a one-day on, two-days off schedule. The control group received the vehicle alone at the same schedule. Working concentrations of RA-9 (10 mg/mL) were reached by dissolution in Cremophor EL and polyethylene glycol 400 (Sigma). Before each injection RA-9 was further diluted in 0.9% saline (working concentration 1 mg/mL). To monitor for tumor growth, RA-9 treated and control mice were imaged with an IVIS SpectrumCT Pre-clinical in vivo imaging system (PerkinElmer) every other day. Animals were sacrificed when abdomens became distended to twice normal size.

**Total blood cell count**

Analysis of total blood cell count from saline- and RA-9 treated mice was performed at the Veterinary Clinical Pathology Laboratory at the University of Minnesota and reviewed by a Board-Certified Clinical Pathologist.

**IHC**

Sections of (3–4 μm) of paraffin-embedded tissues were used for IHC as we have previously described (11). Briefly, following deparaffinization and rehydration, sections were incubated with the mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA) diluted 1:10,000 for 1 hour at room temperature. The avidin—biotin—peroxidase complex method from DAKO was used to visualize antibody binding, and the tissues were counterstained with hematoxylin. For immunohistochemical analyses of mouse tissues, tumors and organs from mice were excised, fixed in 10% neutral buffered formalin, and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed according to standard histologic procedure.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

Sections (3–4 μm) of paraffin-embedded tissues were processed for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using an established method to assay for cell death–associated DNA double-strand breaks as we previously described (11).

**Statistical analysis**

Unless otherwise indicated, results are reported as mean ± SD, and statistical significance was assessed by two-tailed Student t tests using Prism (V.4 Graphpad) with the level of significance set at *P* < 0.05. Survival was summarized using Kaplan–Meier methods and compared using log-rank tests. The combination index (CI) of RA-9 and cisplatin was calculated using the method of Chou and Talaly (15). CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.
Results

RA-9 blocks proteasome-associated DUB activity in ovarian cancer cells

Within the UPS, DUB expression and activity has been linked to cancer initiation, progression, and onset of chemoresistance (7, 8, 21–24). Through multiple iterations of a new class of small-molecule UPS inhibitors, we have recently identified RA-9 (Fig. 1A) as a promising inhibitor that blocks ubiquitin-dependent protein degradation without affecting 20S proteasome proteolytic activity (15). To test whether RA-9 is a DUB inhibitor, we initially evaluated its impact on protein ubiquitination in ES-2 ovarian cancer cell line exposed to 10 μmol/L RA-9 for 4, 8, 16, and 24 hours. Amido black staining was used as loading control. C, quantification of the ubiquitin/total protein (amido) ratio expressed as percentage of t0. D, left, dose-dependent residual DUB activity in 19S RP exposed to the indicated doses of RA-9 expressed as relative fluorescence units (RFU). b-AP15 was used as positive control. Middle, time-dependent residual deubiquitinating enzyme activity in 19S proteasome particles exposed to the indicated dose of RA-9 as expressed as fold RFUs difference over time. b-AP15 was used as positive control. Bortezomib was used as negative control. Right, dose-dependent residual DUB activity in ES-2 cell lysate (total DUBs activity) exposed to the indicated doses of RA-9 expressed as RFUs. b-AP15 was used as positive control.

Next, we monitored the rate of poly-ubiquitinated protein accumulation in ES-2 ovarian cancer cells exposed to 10 μmol/L RA-9 over a period of 24 hours. As shown in Fig. 1B, immunoblot analysis revealed a rapid, time-dependent increase of high-molecular weight poly-ubiquitinated species accompanied by a decrease in mono-, tri-, and tetra-ubiquitin forms. Quantification of the changes in high- and low-molecular weight ubiquitin species in treated cells versus control is given in Fig. 1. Similar results were obtained with SKOV-3 and TOV-12G ovarian cancer cell lines (Supplementary Fig. S1B).

The human genome encodes for at least 98 DUBs, of which only USP14 and UCH37 directly associate with the 19S RP (25). The structural similarities between RA-9 and previously described DUBs inhibitors (9, 26, 27), together with its lack of inhibition of the 20S catalytic activities (15), suggest that RA-9 may selectively inhibit 19S RP-associated DUBs rather than acting broadly on the cellular pool of DUBs as a whole. To test this hypothesis, first we monitored the residual DUB activity in purified 19S RP exposed to
increasing concentration of RA-9 (0–50 μmol/L). As shown in Fig. 1D, RA-9 treatment resulted in a dose- (left) and time-dependent (middle) reduction in 19S RP-associated DUB activity following RA-9 exposure. Second, to determine if RA-9 broadly inhibits cellular DUB activity as a whole, we next measured the total deubiquitinating enzyme activity in cell lysate from ES-2 ovarian cancer cells exposed to escalating doses of RA-9. As shown in Fig. 1D (right), RA-9 treatment does not affect total DUB activity in ES-2 cell lysate. The 19S RP-associated DUB inhibitor b-AP15 and the 20S proteasome inhibitor zopolrestat were used as positive and negative control, respectively. Taken together, these observations suggest that RA-9 is a selective inhibitor of 19S RP-associated DUB activity.

RA-9 inhibits growth of ovarian cancer cell lines and primary cultures

We and others have previously shown that cancer cells exhibit higher endogenous levels of proteotoxic stress inherent to the cancer phenotype (11, 12), as compared with normal cells. This renders ovarian cancer cells more dependent upon ubiquitin-mediated protein degradation than immortalized ovarian surface epithelial cells (11, 12, 28). Therefore, we examined whether inhibition of 19S RP-associated DUBs via RA-9 treatment would selectively cause loss of viability of a panel of ovarian cancer cells as compared with normal ovarian epithelial cells. Specifically, we compared the effect of RA-9 on the viability of the cisplatin-sensitive TOV-21G and ES-2 cell lines, the cisplatin-resistant OVCAR-3 and HEY cell lines, primary ovarian cancer cell cultures (derived directly from clinical isolates of ovarian cancers) versus primary ovarian surface epithelial cell cultures (OSE) isolated from ovaries of women undergoing oophorectomies for benign reasons. Our results indicate that exposure to increasing concentrations of RA-9 over a period of 48 hours compromised the viability of ovarian cancer cells in a dose-dependent fashion (Fig. 2B–D) and to greater extent as compared with OSEs (Fig. 2A). Importantly, although TOV-21G and ES-2 cell lines are traditionally considered cisplatin-sensitive, several studies have recently reported a certain degree platinum resistance (29, 30). Thus, we measured the IC$_{50}$ levels for cisplatin treatment of ES-2 and TOV-21G cells. As shown in Supplementary Fig. S2A, the IC$_{50}$ levels for cisplatin were 11.8 and 13.9 μmol/L for ES-2 and TOV-21G ovarian cancer cell lines, respectively. Furthermore, combination of cisplatin and RA-9 resulted in synergistic effect in terms of loss of cell viability in cisplatin-resistant ovarian cancer cells (Supplementary Fig. S2B).

Because RA-9 inhibits ubiquitin-mediated protein degradation independently from inhibition of the catalytic activities of the proteasomes (15; Fig. 1D), we reasoned that cancer cells that developed resistance to proteasome inhibitors would still remain sensitive to RA-9 treatment. To test this hypothesis, the multiple myeloma (MM) cell lines RPMI8226 and ANBL6 and their bortezomib-resistant derivates RPMI8226-V10R and ANBL6-V10R (16, 31) were exposed to either bortezomib or RA-9 and the residual cell viability measured after 48 hours of treatment. Surprisingly, as shown in Fig. 2E, RA-9 treatment was more effective against bortezomib-resistant MM cell lines than their bortezomib-sensitive parental cell lines.

Taken together, this suggests that the selectivity profile of RA-9 for ovarian cancer cells is dependent upon their levels of proteotoxic stress and independent from their histologic subtype. This also suggests that RA-9 treatment is effective against cancer cells that developed resistance to the licensed proteasome inhibitor bortezomib. Lastly, the synergistic activity of RA-9 combined with cisplatin on the cell viability of ovarian cancer cells suggests that this combinatorial approach may be effective for ovarian cancer treatment, and that RA-9 might still be used to treat cisplatin-resistant disease.

RA-9 causes cell-cycle arrest and caspase-mediated apoptosis in ovarian cancer cells

Ubiquitin-dependent protein degradation regulates the steady levels of key cell-cycle regulatory proteins whose dysregulation is expected to affect cell-cycle progression and viability (32). To test whether the reduced cell viability of ovarian cancer cell lines following RA-9 treatment is associated with cell-cycle dysregulation, ES-2 cells were incubated with increasing concentrations of RA-9 and analyzed by flow cytometry after staining with PI. As shown in Fig. 3A, 18-hour exposure to RA-9 resulted in a dose-dependent increase in the fraction of ES-2 cells in the G$_{2}$–M cell-cycle phase in ES-2–treated cells. To investigate the fate of the cells accumulated in G$_{2}$–M following RA-9 treatment, we analyzed them by flow cytometry after staining with the phycoerythrin (PE)-conjugated antibody specific for the active form of caspase-3, a marker of apoptosis. As shown in Fig. 3B, treatment for 18 hours with RA-9 produced a dose-dependent increase in the levels of active caspase-3. Further, we measured the levels of cleaved PARP, a substrate of caspase-3. ES-2 cells exposed to 5 μmol/L RA-9 for 24 hours were analyzed by Western blot using an antibody recognizing both full-length and cleaved PARP. As shown in Fig. 3C, RA-9 treatment resulted a time-dependent accumulation of the cleaved PARP noticeable as early as 8 hours after RA-9 treatment. Quantification of the ratio between full-length and cleaved PARP in ES-2 cells treated for up to 24 hours is given in Fig. 3D. Taken together, these data suggest that the loss of cell viability by ovarian cancer cell lines following RA-9 treatment is associated with caspase-3–mediated apoptotic cell death.

RA-9 induces UPR in ovarian cancer cells

Activation of UPR, including endoplasmic reticulum (ER) stress responses, is a cellular mechanism to compensate for increasing levels of proteotoxic stress (33, 34) typical of the malignant phenotype and upon proteasome inhibition. We have previously shown that sensitivity to inhibition of ubiquitin-mediated protein degradation in ovarian cancer cells is dependent upon the levels of ubiquitin–proteasome stress.

Because RA-9 treatment resulted in the rapid accumulation of poly-ubiquitinated proteins, we investigated its
effect on ER stress responses in a panel of ovarian cancer cell lines. ES-2, SKOV-3, and TOV-21G cells were exposed to 5 μmol/L RA-9 for 24 hours and subjected to Western blot analysis for the levels of key markers of early and late ER stress responses. As shown in Fig. 4 (left), RA-9 exposure caused a time-dependent increase in the steady levels of the early ER stress markers GRP-78 and PERK, as well as the late ER stress markers IRE1-α and Ero1L-α. This increase was detectable as early as 4 hours following drug exposure, and is consistent with an attempt by the ovarian cancer cells to compensate for RA-9-induced proteotoxic stress by both inhibiting transcription and increasing protein degradation. Quantification of the changes in the steady-state levels of these ER stress markers over time is given in Fig. 4 (right). Furthermore, as shown in Fig. 4B, RA-9 treatment resulted in time-dependent increase in p-eIF2-α levels in ES-2 and TOV-21G ovarian cancer cells, thus strengthening the evidence of a general halting in the cellular translational machinery following drug treatment. On the contrary, we did not observe increase in GCN2 expression levels following RA-9 treatment (Supplementary Fig. S4). Consistent with our previous reports of greater dependency of ovarian cancer cells upon proteolytic degradation machinery (11, 15, 16), exposure of OSEs to RA-9 treatment resulted
in only moderate increase in poly-ubiquitinated protein levels (Supplementary Fig. S3A) and no sign of UPR stress as measured by Ero1L-α expression levels (Supplementary Fig. S3B).

**RA-9 inhibits human ovarian cancer cell growth in vivo and prolongs survival in a mouse model for ovarian cancer**

Having shown that RA-9 treatment induces onset of apoptosis in ovarian cancer cells in vitro, we next investigated the efficacy of RA-9 in inhibiting ES-2 tumor growth in a mouse xenograft model of ovarian cancer. Specifically, NCr nu/nu mice were inoculated i.p. with 100,000 ES-2 ovarian cancer cells stably expressing the GFP reporter with 93% of the animals developing a detectable tumor within 6 days from inoculation. Mice were then randomly assigned to RA-9 treatment group (5 mg/kg, n = 12) or 0.9% normal saline-treated controls (n = 12) and treated by i.p. injections on a 1-day on, 2-day off schedule. The average tumor burden, as measured by fluorescence intensity, at the beginning of the treatment (day 0) was not significantly different in the RA-9 and control cohorts. However, a significant reduction in tumor burden was observed in mice treated with RA-9 versus the control group by day 5 of treatment (Supplementary Fig. S5). The difference became more significant by day 12 (P < 0.002) as measured by fluorescence intensity (Fig. 5A and B). A survival curve for mice in each group is shown in Fig. 5C, with a log-rank test revealing a significant prolongation in overall survival by RA-9 treatment (P < 0.0005). Notably, by day 17 of treatment, all of the control mice were sacrificed due to their tumor burden, whereas only 15% of mice in the RA-9 treatment arm had to be euthanized. Consistent with the reduction in tumor growth and increased overall survival, RA-9–treated mice also showed a reduction in tumor mass (Fig. 5D, left) and in ascitic fluid (Fig. 5D, right) as compared with the control cohort. Next, we tested whether the effect of RA-9 on the increase in ER stress and poly-ubiquitinated protein responses in vitro could be recapitulated in cancer cells derived from saline- and RA-9–treated mice.
tumors from either vehicle- or RA-9–treated mice were collected 8 hours after the last treatment and cell lysate subjected to Western blot analysis for levels of the ER stress response protein GRP-78 and ubiquitin. As shown in Fig. 5E, Western blot analysis revealed an increase in the levels of GRP-78 and high-molecular weight poly-ubiquitinated proteins suggesting that RA-9–mediated anticancer activity in vivo occurs via inhibition of ubiquitin-proteasomal pathway and is associated to ER stress responses activation.

RA-9 caused no significant toxicity on the host

RA-9 was well tolerated in mice at the dose of 5 mg/kg with the difference in total body weight in treated and control cohorts never exceeding 3% (Supplementary Fig. S5). In addition, no statistically significant difference was found in terms of complete blood count and white blood cell (WBC) differential in treated and control cohorts (Fig. 5F). Importantly, no evidence of toxicity was found in treated and control cohorts as assessed by H&E staining on histologic tissue sections of liver, spleen, heart, and kidneys (Fig. 6A).

Next, tumor sections harvested from control and RA-9–treated mice were subjected to H&E or PCNA staining as well as TUNEL assay to evaluate for onset of apoptosis and cell proliferation status. As shown in Fig. 6B, H&E staining revealed a pattern of tumor regression and the presence of apoptotic cells defined by cytoplasmic shrinkage and nuclear chromatin condensation in RA-9–treated versus control cohorts (Fig. 6B). This was accompanied with a marked reduction in cellular proliferation and increased DNA

![Graph showing GRP-78, IRE1-α, and Ero1L-α expression over time for ES-2, SKOV-3, and TOV-21G cells](image)

![Graph showing p-eIF2-α expression over time for ES-2 and TOV-21G cells](image)
fragmentation in tumor sections from RA-9–treated versus control as assessed by PCNA staining (Fig. 6C) and TUNEL assay (Fig. 6D), respectively.

Discussion

Targeting of metabolic pathways to selectively kill cancer cells has promise as an anticancer strategy. The use of the clinically available proteasome inhibitors bortezomib and carfilzomib resulted in improved clinical outcome in patients with MM and mantle cell lymphoma. However, the use of proteasome inhibitors has also been linked to onset of side effects and resistance (13, 14, 35, 36), and bortezomib has had limited activity against solid cancers, including ovarian cancer (37). This has prompt new efforts in the direction of developing second- and third-generation proteasome inhibitors currently in clinical trials or in...
preclinical characterization phase (38). This has also led efforts to generate molecules targeting ubiquitin–proteasome degradation pathways independently from inhibition of catalytic activities of the proteasome.

In this context, we have recently provided evidence that targeting of ubiquitin-dependent protein degradation upstream of the proteasome may represent a novel therapeutic approach for cancer treatment. This includes the DUB inhibitor b-AP15 and RA-190, a small-molecule inhibitor of ubiquitin receptor RPN13 (4, 5, 7, 9, 16, 21, 24, 39). To date, the feasibility of targeting DUBs for ovarian cancer treatment and the effect of DUBs inhibition on ovarian cancer cells in vitro and in vivo are unclear.

During a recent drug development effort screening for small-molecule inhibitors of ubiquitin-mediated protein degradation, we identified two distinct classes of compounds: (i) compounds with selective inhibitory capacity toward the catalytic activities of the 20S proteasome (40) and (ii) compounds that inhibit ubiquitin-mediated protein degradation independently of the 20S catalytic activity, suggesting a possible role as DUB inhibitors (15). In this study, we describe and characterize RA-9 as a small-molecule inhibitor of 19S RP-associated DUBs, its mechanism of action on ovarian cancer cells in vitro and in vivo, and its therapeutic potential for ovarian cancer treatment.

The presence of α-β carbonyl system has been suggested as the molecular determinant for inhibiting DUBs activity via interaction with the cysteine-based catalytic domain found in their active sites (27, 41–43). In this study, we contribute to this view by showing that RA-9 is capable of preventing ubiquitin-mediated protein degradation and free-ubiquitin recycling upstream of the 20S proteolytic activity. Furthermore, although RA-9 treatment resulted in inhibition of DUB activity in affinity-purified 19S RP, it fails to exert the same effect on total DUB activity measured in whole cell lysates. This indicates that RA-9 is not a general DUB inhibitor, but rather an inhibitor of 19S RP-associated DUBs, although we cannot rule out activity against a small...
fraction of other cellular DUBs at this time and further studies of its mechanism are needed.

We and others have previously shown that independently from the genetic mutations leading to cancer, malignant transformation is accompanied with progressive upregulation of the metabolic cancer machinery to sustain the increased proliferation rate that is typical of cancer cells (11, 12, 14). This phenomenon renders ovarian cancer cells selectively sensitive to inhibition of ubiquitin-dependent protein degradation. In this study, we show that inhibition of 19S RP-associated DUBs via RA-9 treatment selectively hinders the cell viability of our panel of primary ovarian cancer cultures and ovarian cancer cell lines, including those resistant to conventional chemotherapy. Indeed, RA-9 effectively inhibits the growth of these chemoresistant ovarian cancer cell lines at pharmacologically achievable doses. Reduction in cell viability following RA-9 exposure occurred in ovarian cancer cell lines containing either wild-type p53 or mutant p53, indicating that ovarian cancer cell sensitivity to RA-9 is independent of p53 mutation status (11). Importantly, RA-9 treatment caused marked reduction in cell viability of MM cells and their bortezomib-resistant parental lines. This strongly suggests that RA-9 could be used in the treatment of multiple cancers, including MM. This may also include forms of cancer resistant to clinically available proteasome inhibitors based upon our in vitro studies of bortezomib-resistant MM lines. Interestingly, the bortezomib-resistant MM lines were both slightly more sensitive to RA-9 than their parental lines.

A major determinant of cell fate is regulation of cell cycle. Previous investigations have shown that DUB substrates include proteins involved in the regulation of the cell-cycle progression (44, 45). In this study, we show that treatment of ovarian cancer cells with RA-9 caused dose-dependent accumulation in G2-M phase within 8 hours of drug exposure. Importantly, our studies also show that failure to proceed through cell cycle leads to loss of cell viability associated with caspase-3 activation and apoptosis.

Activation of UPR is a cellular response to accumulation of unfolded or misfolded proteins in the lumen of the ER (28, 34, 46, 47). The UPR attempts to restore protein homeostasis by both halting translation and increasing protein degradation (48, 49). Here, we show that greater levels of proteotoxic stress caused by RA-9 increase in the steady-state levels of both early and late ER stress response markers, including GRP-78, IRE1-, Ero1L-α, and p-eIF2-α. This suggests that ovarian cancer cells attempt to escape cell death by slowing down protein translation and activating protein degradation in response to RA-9.

In addition to our in vitro studies, we also examined antiovary cancer activity of RA-9 in vivo. Our results show that treatment of ovarian cancer xenograft with RA-9 significantly retarded tumor growth and increased overall survival. Consistent with the effect on UPR induction in vitro, we also observed increase in the levels of ER stress-associated and poly-ubiquitinated proteins in ES-2 tumor cells of treated mice. As seen for in vitro studies, this was accompanied by marked reduction in cellular proliferation and onset of apoptosis in tumor sections from RA-9-treated versus control. Importantly, our data show that RA-9 treatment was not associated with significant toxicity, difference in body weight, blood profile or histology of major organs as observed between RA-9-treated and control mice. Collectively, our findings suggest that RA-9 has significant in vitro and in vivo anticaner activity at doses well tolerated in a mouse xenograft model, and warrants further exploration as a therapeutic agent for ovarian cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The funding agencies had no role in study design, data collection and analysis, decision to publish or preparation of the article.

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Small-Molecule RA-9 Inhibits Proteasome-Associated DUBs and Ovarian Cancer *In Vitro* and *In Vivo* via Exacerbating Unfolded Protein Responses

Kathleen Coughlin, Ravi Anchoori, Yoshie Iizuka, et al.

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