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Romidepsin (FK228) combined with cisplatin stimulates DNA damage-induced cell death in ovarian cancer

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Abstract

Objective—Romidepsin (FK228) was recently approved by the FDA for the treatment of cutaneous and peripheral T cell lymphoma. We have shown in vitro efficacy of FK228 in ovarian cancer. Here, our goal was to evaluate FK228 combined with cisplatin in ovarian cancer in vitro and in vivo.

Methods—Ovarian cancer cell lines were treated with cisplatin, FK228 or the combination of drugs. Colorimetric assays were used to determine cytotoxicity in vitro. Mice engrafted with 7×10^6 SKOV-3 ovarian cancer cells were treated with cisplatin, FK228 or the combination, and tumor weights and volumes were measured. We assessed molecular markers of proliferation (mib-1), apoptosis (cleaved PARP and cleaved caspase 3) and DNA damage (pH2AX, RAD51 and 53BP1).

Results—FK228 enhanced the cytotoxic effects of cisplatin in ovarian cells compared to vehicle-treated controls or each drug alone. The combination of FK228 and cisplatin- induced apoptosis and activated aberrant DNA damage responses demonstrated by increased expression of pH2AX, RAD51 and 53BP1. Mice treated with FK228, cisplatin and both drugs reduced tumor weights and volumes. Drug-treated tumors showed decreased mib-1 and increased cleaved-caspase 3 expression levels. The number and intensity of pH2AX stained cells was greatest in tumors exposed to the combination of FK228 and cisplatin.

Conclusion—FK228 causes DNA damage-induced apoptosis and enhances the anti-tumor effects of cisplatin. The DNA damage mark pH2AX is activated by FK228 and may be a useful pharmacodynamic mark of these effects.

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Keywords

Romidepsin; depsipeptide (FK228); histone deacetylase inhibitors (HDACi); pH2AX; ovarian cancer

Introduction

Ovarian cancer remains the deadliest gynecologic cancer in the United States [1]. Current treatment includes surgical staging and tumor debulking followed by platinum-based chemotherapy [2]. The majority of high-grade serous ovarian tumors harbor defects in DNA damage repair pathways [3] and are theoretically more sensitive to the effects of DNA damaging drugs such as cisplatin. An emerging treatment strategy is to target altered DNA damage response mechanisms in susceptible tumor cells with poly (ADP-ribose) polymerase inhibitors that induce DNA damage and synergize with cisplatin [4]. Another approach under investigation by our group is to target chromatin-modifying enzymes associated with DNA damage and repair with small molecule histone deacetylase inhibitors (HDACi).

Our group recently published that HDACi with in vitro efficacy in ovarian cancer cells inhibit cell viability and induce apoptosis [5]. We also showed that of the 18 HDACi compounds we examined, romidepsin (FK228) was the most potent. FK228 was isolated from *Chromobacterium violaceum* no. 968, a rare Gram negative bacterium [6]. FK228 has a cyclic depsipeptide structure, but the main mechanism of action is the reduction of a characteristic disulfide bond that creates a “warhead” thiol group. The thiol binds to zinc in the catalytic center of HDAC proteins and inhibits enzymatic activity [6]. FK228 is currently approved for treating cutaneous and peripheral T-cell lymphomas [7, 8]. However, its role in the treatment of solid tumors, including ovarian malignancies remains under investigation.

FK228 treatment at nanomolar concentrations induces robust and prolonged activation of pH2AX, which strongly correlates with decreased cell viability and apoptosis in ovarian cancer cells [5]. It is well-known that pH2AX is an early and sensitive mark of DNA double-strand breaks and other mechanisms of DNA damage [9]. Sustained induction of pH2AX is associated with irreparable DNA damage and ultimate cell death [10]. Therefore, pH2AX activation may contribute to drug-induced cytotoxicity and function as a surrogate mark of response. We have shown that other HDACi enhance the effects of the DNA damaging agent cisplatin, even in cisplatin-resistant ovarian cancer cells [5]. In this study, we evaluated the combinatory effects of FK228 with cisplatin.

Here, we demonstrate that FK228 enhances the cytotoxic effects of the DNA damaging agent cisplatin in vitro and in vivo. Furthermore, FK228 combined with cisplatin causes robust and prolonged activation of pH2AX and other DNA damage marks. Our results indicate that DNA damage-induced apoptosis is a potential mechanism for the reduction in cell viability and growth observed after exposure to FK228 and cisplatin, and the DNA damage mark pH2AX may be a useful pharmacodynamic mark of these effects.

Materials and Methods

Cell culture and compounds

The epithelial ovarian cancer cell lines SKOV-3, UWB1.289+BRCA1 wild type (Brca1 WT) and UWB1.289 BRCA1 null (Brca1 Null) cell lines (American Type Culture Collection, Manassas, VA), OVCAR-8 and NCI/ADR-RES (National Cancer Institute, Bethesda, MD) were maintained in culture as previously described [5, 11]. The SKOV-3,

OVCAR-8 and NCI/ADR-RES cell lines are represented in the National Cancer Institute 60 Cancer Panel [12–14], and the BRCA1 WT and BRCA Null cell lines have been well described [15]. The cell lines summarized in Supplementary Table 1 were used within 6 months of receipt and tested negative for mycoplasma. Cells were treated with romidepsin (FK228) (Gloucester Pharmaceuticals, Celgene Corporation, Cambridge, MA); cisplatin (Sigma Chemical Company, St Louis, MO); the combination of drugs; or 0.01% dimethyl sulfoxide, DMSO (Sigma) as vehicle controls.

Cell proliferation and cytotoxicity assays

Sulphorhodamine B (SRB) assays were used to determine cell proliferation and cytotoxicity and were conducted as previously described [16], with minor modifications for seeding at a density of 2000 cells/well in 384-well plates (Corning Life Sciences, Lowell, MA). Absorbance was measured at 510nm using a Spectramax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) in the High-Throughput Screening Core of the Vanderbilt Institute of Chemical Biology. Exposure to the cytotoxic compounds results in a reduction of cellular proliferation at the end of the specified time compared to the control cells at time zero where no cytotoxic substance was added.

The interaction between fixed ratios of FK229 and cisplatin was assessed with the Combination Index (CI) method [17]. Synergy, additivity and antagonism between drug combinations is defined as $CI < 0.9$, $CI = 0.9–1.1$ and $CI > 1.1$, respectively, at the effective dose (ED) for 50%, 75% and 90% fractional effects. Light microscopy was performed to assess the morphological effects of treatment.

Animals

Six to eight-week-old female athymic Nude-Foxn1^{nu} mice (Harlan Laboratories, Indianapolis, IN) were purchased after the research protocol was approved by the Vanderbilt University Animal Use and Care Committee. The animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. For this subcutaneous (SC) xenografts model, 7×10^6 SKOV3 tumor cells in a 200 μ L of mixture of PBS and Matrigel (1:1 v/v) (Sigma) were injected SC into the flanks of mice. After the tumors were approximately 1cm³, we treated the mice in cohorts as follows: vehicle control (n=8), cisplatin (n=10), FK228 (n=10) and FK228 with cisplatin (n=10). The drugs were administered via intraperitoneal injection with vehicle (0.01% DMSO in PBS) twice a week; FK228 (1mg/kg) twice a week, cisplatin (5mg/kg) weekly, and the combination of FK228 and cisplatin at the same drug concentrations and frequency as single agent treatment.

Animals were examined for the effects of tumor burden and tumor growth, biweekly, and tumor measurements were performed weekly. Tumor volume was calculated using the formula: tumor volume = $[\text{length} \times \text{width}^2] / 2$ as previously reported [18]. After 2 weeks of treatment and 24 h after the final dose of drug, the mice were euthanized according to protocol and necropsy was performed. Tumors were excised and processed as below.

Western Blotting

Whole cell protein isolation, hydrochloric acid extraction of histones, Western Blotting and signal detection were performed as described [11, 19]. Anti-phospho H2AX (Ser139) and anti-histone H3 (Millipore) were used. Additional antibodies used were anti-PARP (Cell Signaling Technology, Danvers, MA) anti-cleaved caspase 3 (Cell Signaling) and anti- β -actin (Sigma). Loading controls were anti-histone H3 (Millipore) and β -actin for the histones and total proteins, respectively.

Immunofluorescence

After treatment with FK228, cisplatin, or the combination, ovarian cancer cells were fixed, permeabilized and stained with anti-phospho H2AX (Ser139) (pH2AX) (Millipore), RAD51 (Millipore), and 53BP1 (Bethyl Laboratories, Montgomery, TX) as per established protocols [5, 20]. Binding of the primary antibodies was detected with Alexa Fluor anti-mouse IgG 594 or Alexa Fluor anti-rabbit IgG 488 secondary antibodies (Invitrogen, Carlsbad, CA), as appropriate. Images were acquired and analyzed as previously described [5, 20]. We modified this protocol for immunofluorescence staining of the tissues samples (Supplemental Methods).

Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Five micron sections were obtained and adhered to slides and deparaffinized. Hematoxylin and eosin staining for histology and immunostaining was performed in the Vanderbilt Immunohistochemistry Core Facility. Additional details are in Supplemental Methods. Visualization and acquisition of images were performed as published [5] [20].

Statistics

Unless otherwise indicated, values shown were the mean + SE of three independent experiments with * $p < 0.05$ relative to control using the Student's *t* test.

Results

FK228 enhances the cytotoxic effects of cisplatin in vitro

Our group and others have shown that nanomolar concentrations of FK228 effectively reduce cell viability in ovarian cancer cells [5, 11, 21, 22]. Here, we evaluated the anti-proliferative and cytotoxic effects of FK228 (1–10 nM), cisplatin (0.5–5 μ M) and the combination by conducting SRB cellular assays 72 h after drug exposure (Fig 1A). In 4 of the 5 cell lines, FK228 inhibited cell proliferation and viability and enhanced the effects of cisplatin, particularly at lower drug concentrations. CI results demonstrated that the combined drug treatments were synergistic, with CI levels of < 1.0 [17]. SKOV-3, OVCAR-8 and Brca1 Null cells displayed the greatest combinatory effects with CI levels well below 1.0 (Fig 1B). NCI/ADR-Res cells are a multidrug-resistant cell line derived from OVCAR-8 cells and as expected were resistant to both drugs [5]. As a result, the CI results could not be calculated. The Brca1 WT cells were relatively resistant to cisplatin, but sensitive to FK228 compared to the Brca1 Null cells. Because FK228 and cisplatin displayed a similar growth pattern of inhibitory effects, but were synergistic when combined in SKOV-3 cells, we chose these cells for further study. We examined the morphology of SKOV-3 cells by light microscopy at 72 h and found a decrease in the number of viable cells with each drug compared to control that was reduced further by combination treatment (Fig 1C). In addition, in the drug-treated groups we observed an increase in round pyknotic cells consistent with apoptosis.

To confirm that apoptosis contributed to the observed cytotoxic effects, we probed for the expression of cleaved PARP and caspase 3 by Western Blot in SKOV-3 cells treated with FK228, cisplatin and the combination at 24 h, a time point earlier than the cytotoxic effects seen by SRB (Fig 1D). Both cleaved PARP and cleaved caspase 3 were activated by the combination of FK228 and cisplatin, compared to vehicle-treated controls and each drug alone.

FK228 combined with cisplatin activates p_H2AX and other DNA damage marks more than each drug alone

Persistent p_H2AX activation, greater than 24 h, is associated with DNA damage-induced apoptosis [10]. We have demonstrated previously that p_H2AX is enhanced by the combination of HDACi and DNA damaging agents [5, 21], but did not specifically test the effects of FK228 combined with cisplatin. Here, Western Blot analysis of SKOV-3 treated cells showed greater p_H2AX expression with combination treatment compared to controls and to each drug alone (Fig 2A). Furthermore, the number of foci and levels of pan-nuclear foci and saturated staining of p_H2AX detected by IF were significantly upregulated in the presence of both FK228 and cisplatin ($p < 0.0001$) (Figs 2B).

Next, we examined other marks of DNA damage response, RAD51 and 53BP1 by IF staining. We found that the number of foci and intensity of both RAD51 and 53BP1 were enhanced by the combination of FK228 and cisplatin and both proteins co-localized with p_H2AX (Fig 3A & B).

FK228, alone and combined with cisplatin, reduces ovarian cancer tumor growth and activates p_H2AX in vivo

Treatment with FK228, cisplatin and the drugs combined was well tolerated by all of the mice. At the completion of the study, tumors were extracted and weighed (Fig. 4A). The combined tumor weights in each group were not significantly smaller in the treated mice compared to controls, except for the FK228-treated tumors ($p = 0.0214$) (Fig 4–B). However, longitudinal tumor measurements showed that tumor volume was reduced by FK228, cisplatin and the combination of drugs compared to vehicle-treated controls cisplatin ($p = 0.015$); FK228 ($p = 0.008$); FK228 + cisplatin ($p = 0.0045$) (Fig. 4C). Furthermore, a Waterfall plot demonstrated that the rate of tumor growth was slower in mice treated with the combination of FK228 and cisplatin (Fig. 4D).

We chose a 2-week time course in order to detect early differences in the molecular response to treatment. We stained extracted tumors for markers of cell proliferation (mib-1) and apoptosis (cl-caspase 3). The expression of mib-1 (by number of tumor cells and intensity) was lower in tumors exposed to FK228, cisplatin and the combination compared to controls (Fig 5A). Conversely, the number of cells with cl-caspase 3 was higher in treated tumors (Figs. 5B). Finally, we evaluated the expression of p_H2AX in the tumor samples by IF staining (Fig 6). FK228 combined with cisplatin enhanced p_H2AX diffuse nuclear staining from 8.53% to 26.19% ($p = 0.025$).

Discussion

In this report, we have shown that FK228 enhances the cytotoxic effects of cisplatin in vitro. FK228, alone and combined with cisplatin, reduces ovarian cancer tumor growth in vivo. Furthermore, FK228 activates the expression of p_H2AX and other DNA damage marks when combined with cisplatin. Our group has shown previously that FK228 is a potent inhibitor of ovarian cancer cell growth in vitro [5, 11, 21, 22] and leads to robust induction of the DNA damage mark p_H2AX [5, 21]. Persistent p_H2AX activation, greater than 24 h, is associated with DNA damage associated cell death [10]. In cells treated with FK228 and cisplatin, p_H2AX is further upregulated. This finding suggests a mechanism for how FK228 enhances cisplatin DNA damage-induced cell death.

In this study, FK228 combined with cisplatin increased the number of discrete nuclear foci as well as diffuse pan-nuclear saturation of p_H2AX. The significance of pan-nuclear saturation suggests replication-associated DNA damage or TRAIL-induced cell death [9]. FK228 is a potent inhibitor of class I HDACs (1, 2 and 3) [6]. Previous studies have shown

that inhibition of class I HDACs induces DNA damage and activation of p_H2AX and both homologous recombination and nonhomologous end joining mechanisms of DNA damage response [23–25]. Here, we show that other DNA damage response marks RAD51 and 53BP1 are activated by FK228 alone and with cisplatin. This suggests that FK228 alters multiple mechanisms of DNA repair.

Our group and others have demonstrated that several types of HDACi compounds enhance the cytotoxic effects of cisplatin and other DNA damaging agents [5, 21, 26]. However, there are differences in HDACi cytotoxicity depending on the type of drug combination [27–30]. Some HDACi are preferentially synergistic with DNA damaging agents, but do not augment metabolic antagonists or microtubule targeting drugs such as Taxol [26]. Thus determining optimal combination therapy will depend on the specific HDACi agent, the features of the combination agent and careful selection of the susceptible tumor type.

Careful selection of tumor sub-types is highly relevant for HDACi treatment. Of the ovarian cancer cell lines examined by our group, only NCI/ADR-Res cells (with known resistance to multiple anti-tumor agents) are completely resistant to FK228 [5]. NCI/ADR-Res express high levels of the ABC transporter multi-drug resistance gene, MDR1 [14]. FK228 is known to induce PgP [31–33]. Thus, it is possible that further upregulation of PgP contributes to relative resistance to FK228. Upregulation of MDR1 with concomitant downregulation of other ABC transporter genes has been observed with HDACi treatment [34–38]. In ovarian cancer cells, certain HDACi downregulate the ABC transporter MRP2 and contribute to cellular sensitivity to chemotherapy [34]. We have shown that NCI/ADR-Res ovarian cancer cells are sensitive to other types of HDACi drugs, and resistance to cisplatin is overcome with the combination of SAHA and cisplatin [5]. This suggests that the efficacy of treatment with HDACi depends on tumor/cell type in the context of specific properties of HDACi drugs. Mechanisms of cellular resistance and response are important areas under investigation by our group and will affect the selection of tumor types most appropriate for FK228-based therapy. We acknowledge the limitations of the small number of cell lines evaluated in this report and are planning future studies to expand our sample size that will also include a variety of normal cell types.

In conclusion, FK228, alone and in combination with cisplatin, reduces cell growth in vitro and in vivo in susceptible ovarian cancer cells. Furthermore, p_H2AX, a key sensor of DNA damage, is activated by FK228 alone and combined with cisplatin. Prolonged and robust activation of p_H2AX is associated with DNA damage-induced cell death in the presence of FK228. The aberrant induction of p_H2AX implies a potential mechanism for the observed cytotoxic effects of the combined drugs, and could be a useful pharmacodynamic marker of treatment response. These preclinical studies and our previous work [5, 11, 21, 22] provide a foundation for future clinical evaluation of FK228 combined with a platinum-based regimen in the setting of recurrent ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- FK228 combined with cisplatin augments DNA damage-induced apoptosis in ovarian cancer cells
- The DNA damage response protein pH2AX is activated by FK228 and cisplatin in vitro and in vivo
- pH2AX may be a useful pharmacodynamic mark of FK228-based therapy

Figure 1. FK228 reduces cell viability alone and combined with cisplatin in ovarian cancer cells
 A) Ovarian cancer cells (SKOV-3, OVCAR-8, NCI/ADR-Res, UWB1.289 BRCA1 null and UWB1.289+BRCA1 wild-type) were treated with FK228 (1–20nM), cisplatin (0.5–10uM) or both. SRB assays were performed to assess cytotoxicity and cell viability after 72 h of treatment. Each dose was replicated 6 times. Values are shown as the mean + SE for three independent experiments. B) CI results for the drug treatments are shown. A CI of < 1.0 is considered to be synergistic. C) Illustrative light microscopy (10X) of SKOV-3 cells showing cellular morphology. The arrow points to two round pyknotic cells. D) Representative Western blot analysis of apoptotic markers cleaved PARP and cleaved caspase 3. The loading control was β -actin.

Figure 2. FK228 combined with cisplatin increases the expression of the DNA damage mark pH2AX in vitro

SKOV-3 ovarian cancer cells were treated with cisplatin (5 M), FK228 (10 nM) or the combination for 24 h and evaluated by Western blot and IF staining for the expression of pH2AX. A) Western blot evaluation of pH2AX. Histone H3 was the loading control. B) IF nuclear stain for pH2AX (green). White arrow points to discrete foci, which are characteristic of pH2AX activation at sites of DNA double stranded breaks. The number of nuclei displaying 1, 2–5, greater than 5 foci; or diffuse nuclear staining for pH2AX foci was quantified and is presented in percentages. Values are mean + SE for 3 independent high-power fields of 100 cells each.

Figure 3. pH2AX co-localizes with other DNA damage response marks RAD51 and 53BP1 in treated SKOV-3 ovarian cancer cells treated with vehicle, cisplatin, FK228 or the combination for 24 h

Representative images (40X) of IF staining for: A) pH2AX (red) and RAD51 (green); and B) pH2AX (red) and 53BP1 (green). DAPI (blue) stained for nuclear DNA.

Figure 4. FK228 inhibits tumor growth alone and in combination with cisplatin in vivo
 A) Representative images of the extracted tumors after treatment with cisplatin (5mg/kg weekly), n=10; FK228 (1mg/kg twice weekly), n=10; or both drugs at the same doses and frequency as the single agents, n=10; compared to vehicle controls (0.01%DMSO twice weekly), n=8. B) Tumor weights (g) after the completion of treatment. C) Tumor volume (mm³) measurements over the 2-week course of treatment. D) Waterfall plot of tumor volume change (%). Each column represents one individual mouse, with data expressed relative to the pre-treatment tumor volume.

Figure 5. FK228 combined with cisplatin reduces the levels of mib-1 and increases cl-caspase 3 in vivo

A) Representative images (40X) of mib-1 staining in tumors treated with vehicle control, cisplatin, FK228 or cisplatin combined with FK228. B) Representative images (40X) of cl-caspase 3 staining in tumors treated as above.

Figure 6. FK228 and cisplatin induce the DNA damage mark pH2AX in SKOV-3 tumors in vivo In tumors treated with vehicle, cisplatin, FK228 and the combination, pH2AX was evaluated by IF. A) Representative images of IF staining for pH2AX at 40X (green). DAPI (blue) staining indicates the nuclear stain. B) Quantification of IHC staining of pH2AX. Values are mean + SE for 3 independent high-power fields of 100 cells each.