VLX600 Disrupts Homologous Recombination and Synergizes with PARP Inhibitors and Cisplatin by Inhibiting Histone Lysine Demethylases

Thomas L. Ekstrom¹, Nicholas M. Pathoulas¹, Amelia M. Huehls¹, Arun Kanakkanthara¹,²*, and Larry M. Karnitz¹,²*

Division of Oncology Research¹ and Department of Molecular Pharmacology and Experimental Therapeutics², Mayo Clinic College of Medicine, Mayo Clinic, Rochester, MN, 55905

Running Title: VLX600 synergizes with PARP inhibitors and cisplatin

Keywords: High-grade serous ovarian cancer, poly(ADP-ribose) polymerase, VLX600, RAD51, histone lysine demethylases

Abbreviations: Poly(ADP-ribose) polymerase (PARP); PARP inhibitor (PARPi), Histone lysine demethylases (KDM); High-grade serous ovarian cancer (HGSOC), Oxidative phosphorylation (OXPHOS)

To whom correspondence should be addressed:
Larry M. Karnitz or Arun Kanakkanthara, Division of Oncology Research, Gonda 19-300, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Telephone: 507-284-3124; Email: karnitz.larry@mayo.edu, kanakkanthara.arun@mayo.edu

Competing interests: A. Kanakkanthara and L. M. Karnitz are co-inventors on a provisional patent application related to the findings in this manuscript. The other authors declare no potential conflicts of interest.
Abstract

Tumors with defective homologous recombination (HR) DNA repair are more sensitive to chemotherapies that induce lesions repaired by HR as well as poly(ADP-ribose) polymerase (PARP) inhibitors (PARPis). However, these therapies have limited activity in HR-proficient cells. Accordingly, agents that disrupt HR may be a means to augment the activities of these therapies in HR-proficient tumors. Here we show that VLX600, a small molecule that has been in a phase I clinical trial, disrupts HR and synergizes with PARPis and platinum compounds in ovarian cancer cells. We further found that VLX600 and other iron chelators disrupt HR, in part, by inhibiting iron-dependent histone lysine demethylases (KDM) family members, thus blocking recruitment of HR repair proteins, including RAD51, to double-strand DNA breaks. Collectively, these findings suggest that pharmacologically targeting KDM family members with VLX600 may be a potential novel strategy to therapeutically induce HR defects in ovarian cancers and correspondingly sensitize them to platinum agents and PARPis, two standard-of-care therapies for ovarian cancer.
**Introduction**

Ovarian cancer, especially the most common high-grade serous ovarian cancer (HGSOC), is the leading cause of death for gynecological cancers in women (1,2). Upwards of 50% of these tumors have defects in homologous recombination (HR) DNA repair due to mutations in or epigenetic silencing of *BRCA1*, *BRCA2*, and other genes involved in HR (3). HR defects are associated with better survival following platinum-based chemotherapy (4,5), likely due to the fact that platinum therapies induce lesions that are repaired by HR (6,7). Similarly, cancer cells with HR defects are more sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors (6), which block PARP catalytic activity and trap PARP complexes on DNA, ultimately causing cell death (8-10). Based on their activity, these agents are now standard-of-care therapies for HGSOC that exhibit the greatest effects in HR-deficient HGSOCs (11). However, because platinum compounds and PARPis are less active in HR-proficient tumors, strategies to pharmacologically inhibit HR are needed to enhance their activities in HR-proficient HGSOCs.

Chromatin structure, which plays critical roles in DNA repair (12-14), is regulated by posttranslational modifications such as methylation, acetylation, ubiquitylation, and phosphorylation, all of which primarily occur on the tails of histones H2A, H2B, H3, and H4. Recent studies demonstrated that histone methylation and demethylation play complex roles in HR repair, and in some cases both events occur on the same histone residue (15), possibly reflecting the need to repress DNA transcription and sequentially recruit different repair complexes to sites of DNA damage. Consistent with these findings, multiple members of the histone lysine demethylase (KDM) superfamily have been shown to regulate HR (14,16,17). These observations raise the possibility that KDM inhibitors, which are emerging as potential
anticancer agents due to their ability to suppress transcription to allow repair (18,19), may represent a strategy to disrupt HR in cancer cells to increase the cytotoxic activity of DNA crosslinking agents and PARPis.

VLX600 was originally discovered in a screen to identify compounds that were cytotoxic to metabolically stressed tumors (20). The agent had anticancer activity in human tumor xenograft models with little systemic toxicity. Additional studies showed that it was an iron chelator that inhibited mitochondrial oxidative phosphorylation (OXPHOS), and its ability to inhibit OXPHOS was associated with cytotoxicity in metabolically stressed cells (21). These findings led to a phase I trial in humans with cancer in which the maximum-tolerated dose was not reached, the drug was well tolerated, and stable disease was seen as the best response (22).

Here we report that VLX600 disrupts HR repair by inhibiting KDM family members that belong to the Fe$^{2+}/\alpha$-ketoglutarate-dependent dioxygenase KDM families. Consistent with the HR defect induced by VLX600, we show that this agent synergizes with PARPis and cisplatin in killing HR-proficient ovarian cancer cells, raising the possibility that VLX600 may represent a strategy to convert HR-proficient HGSOCs into HR-deficient tumors and thereby increase the activity of standard-of-care therapies, including platinum compounds and PARPis, used to treat this disease.
Materials and Methods

Cell Lines, Cell Culture, Drugs, siRNAs, Plasmids, and siRNA Transfections

OVCAR-8 cells (D. Scudiero, National Cancer Institute, Frederick, MD), OVCAR-8-DR-GFP (23), OV90 (Scott Kaufmann, Mayo Clinic), PEO1 (Scott Kaufmann, Mayo Clinic), and PEO14 (Sigma-Aldrich, St. Louis, MO) were maintained in RPMI-1640 media (Corning, NY) supplemented with 8% fetal bovine serum (EMD Millipore, Burlington, MA). Cell cultures were incubated in a humidified 5% CO₂ incubator at 37°C. All cells were authenticated by autosomal STR profiling (University of Arizona Genetics Core) and were free of Mycoplasma contamination as determined testing with a MycoAlert Mycoplasma Detection Kit (Cat. No. LT07-118, Lonza, Basel, Switzerland). Cell lines were reinitiated every 3-6 months from cryopreserved stocks.

VLX600, RI-1, ciclopirox, and deferoxamine were obtained from Cayman Chemical (Ann Arbor, MI). Olaparib, veliparib, and mirin were obtained from Selleck Chemicals (Houston, TX). Cisplatin and carboplatin were obtained from Teva Pharmaceutical Industries (Petah Tikva, Israel) and Fresenius Kabi (Bad Homburg, Germany), respectively. Plasmids were obtained as follows: pcDNA3 (Invitrogen, Carlsbad, CA), EGFP-N1 (Clontech/Takara Bio, Mountain View, CA), and pCBAScel was a gift from Maria Jasin (Cat. No. 26477, Addgene, Watertown, MA) (24). All siRNAs were purchased from Dhharmacon/Horizon (Layfette, CO) and sequences are listed in Supplementary Table S1. An siRNA that targets firefly luciferase was used as non-targeting siRNA control. For siRNA transfections, 6 – 8 × 10⁶ cells were suspended in a 4-mm cuvette in 180 μL growth media, mixed with 20 μL of 20 μM siRNA per transfection, electroporated using a BTX ECM 830 electroporator using two 280-volt, 10-msec pulses. Transfections were repeated 24 hours later, and cells were used for experiments 48 hours after the second transfection.
unless indicated otherwise. The efficiency of the siRNA knockdown was determined by qRT-PCR and Western blotting as described below.

**Clonogenic Assays**

Cells were plated at 300 (OVCAR-8), 500 (PEO14), 600 (OV90) or 750 (PEO1) cells per well in 6-well plates in triplicate, incubated for 4 hours, treated with vehicle or agents as indicated, cultured for 8-14 days in the continued presence of agents and stained with Coomassie Brilliant Blue. Colonies were manually counted. Survival is expressed as a percentage of the control with no drug treatment. Combination index values were calculated using the Chou-Talalay method (25) and Calcusyn software (Biosoft, Cambridge, England). For the siRNA-mediated knockdown clonogenic assays, cells were plated at the following densities: 1,200 cells/well for siBRCA1 and 300 cells/well for all other siRNAs.

**Direct Repeat Green Fluorescent Protein (DR-GFP) Reporter HR Assay**

\[1 \times 10^7\] OVCAR-8-DR-GFP cells were suspended in 150 μL media in a 4-mm cuvette and mixed with 40 μg pcDNA3 (empty vector control); 5 μg pEGFP-N1 plus 35 μg pcDNA3 (positive control for transfection); or 20 μg pcβAScel plus 20 μg pcDNA3 dissolved in 50 μL RPMI-1640 (without fetal bovine serum) for a total of 40 μg of plasmid per transfection using the same electroporation parameters as described for siRNA transfections. Cells were plated in 6-well plates (100,000 cells/well), treated with the indicated agents for 2 hours after plating, incubated for 72 hours, trypsinized, washed twice in PBS, resuspended in 2% paraformaldehyde in PBS for 5 minutes in the dark at room temperature, washed once with growth media,
resuspended in 1 mL growth media, and analyzed via flow cytometry to determine GFP expression. Percent GFP expression is given as a percentage of the control with no drug treatment. For DR-GFP reporter assays with siRNA-mediated knockdowns, cells were transfected with siRNA, incubated for 24 hours, and then co-transfected with I-SceI expression plasmid plus siRNA, incubated for 72 hours, and analyzed as described above.

**Indirect Immunofluorescence**

For immunostaining of γH2Ax, BRCA1, and RAD51, cells were plated (10,000 cells/well) on 8-well Nunc™ Lab-Tek™ II Chamber Slide™ System (Cat. No. 154534, Thermo Fisher Scientific, Waltham, MA) slides, cultured overnight, and the indicated agents were added. After 2 hours, the cells were exposed to 2 Gy ionizing radiation, and incubated for additional 6 hours. The cells were then washed twice with PBS and fixed with 3% paraformaldehyde in PBS for 15 minutes at room temperature. Slides were washed twice with 0.2% Triton X-100 in PBS, incubated in 0.2% Triton X-100 in PBS for 10 minutes, washed three times with PBS, blocked with 5% bovine serum albumin in PBS containing 0.2% Tween-20 for 30 minutes, and incubated overnight with antibodies to BRCA1 (1:350, Cat. No. sc-6954, Santa Cruz Biotechnology, Dallas, TX), γH2Ax (1:350, Cat. No. A700-053, Bethyl Laboratories, Montgomery, TX), or RAD51 (1:350, Cat. No. PC-130, EMD Millipore) plus γH2Ax (1:350, Cat. No. 05-636, EMD Millipore) in blocking solution. Cells were then washed with PBS and incubated with secondary HRP-linked, anti-rabbit or mouse IgG (1:500) in blocking solution at room temperature for 1 hour. 4’,6-diamidino-2-phenylindole (DAPI) counterstain was used. Images were taken using a Zeiss LSM780 confocal microscope equipped with 40x or 100x objective. Immunostaining for RPA32
was performed as described with the following modifications (26): incubated cells for 4 hours post IR (2 Gy), incubated cells for 15 min in extraction buffer 1, and extraction buffer 2 was not used. Antibodies used were mouse RPA32 (3 µg/ml, Cat. No. NA18-100UG, EMD Millipore) with previously mentioned rabbit γH2Axl (26).

**Western Blotting**

Cell lysate were prepared by lysing cells in ice-cold lysis buffer (50 mM HEPES, 1% Triton X-100, 10 mM NaF, 30 mM Na₄P₂O₇, 150 mM NaCl, 1 mM EDTA, containing freshly added 10 mM β-glycerophosphate, 1 mM Na₃VO₄, 20 µg/ml pepstatin A, 10 µg/ml aproptinin, 20 µg/ml leupeptin, 40 µM microcystin-LR). Lysates were centrifuged at 14,000 rpm for 5 minutes at 4°C, and clarified supernatants were transferred to clean microcentrifuge tubes. Histone extracts were prepared via supplier’s protocol (Cat. No. OP-0006, EpiGentek, Farmingdale, NY). Protein concentrations were determined using the Bio-Rad Bradford Assay (Cat. No. 5000006). Bovine serum albumin (Cat. No. A-420-250, GoldBio, St Louis MO) was for the standard curve. Protein (30-50 µg) was fractionated by Bio-Rad 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Cat. No. 4561094) and transferred to PVDF immobilon-P (Cat. No. IPVH00010, EMD Millipore) membrane. Primary antibodies were rabbit polyclonal RAD51 (1:2,000, Cat. No. PC-130, EMD Millipore), mouse monoclonal β-actin (1:2,000, Cat. No. ab8226, Abcam, Cambridge, UK), rabbit monoclonal H3K9me3 (1:5,000, Cat. No. 13969, Cell Signaling, Danvers, MA), rabbit monoclonal Histone H3 (1:5,000, Cat. No. 4499, Cell Signaling), mouse monoclonal BRCA1 (1:2000, Cat. No. sc-6954, Santa Cruz Biotechnology), mouse monoclonal RPA32 (1:1,000, Cat. No. NA18-100UG, EMD Millipore), rabbit monoclonal KDM4A (1:1,000, Cat. No. 5328, Cell Signaling), rabbit
monoclonal KDM4B (1:1,000, Cat. No. 8639, Cell Signaling), rabbit polyclonal KDM4C (1:1,000, Cat. No. A300-885A, Bethyl Laboratories), rabbit polyclonal H3K4me3 (1:5,000, Cat. No. 07-473, EMD Millipore), rabbit monoclonal H3K36me2 (1:5,000, Cat. No. 2901, Cell Signaling). Rabbit (Cat. No. 7074) and mouse (Cat. No. 7976) horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling. Western blot membranes were developed with SuperSignal chemiluminescent substrate (Cat. No. 34579, Thermo Fisher Scientific), and chemiluminescence was detected using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

**Quantitative real-time PCR**

Total RNA was extracted from cells using an miRNeasy mini kit (Cat. No. 217004, Qiagen) following the supplier’s instructions. cDNA was synthesized from 1 µg of total RNA using oligo(dT) primers and SuperScript™ III reverse transcriptase (Cat. No. 18080-044, Thermo Fisher Scientific). Quantitative real-time PCR was performed in triplicate for each sample using 25 ng cDNA template in a final volume of 20 µL on a CFX96 real-time PCR system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Cat. No. 1725120, Bio-Rad). GAPDH was used to normalize mRNA expression. All qPCR primers were purchased from Integrated DNA Technologies (IDT) and are listed in Supplementary Table S2.

**Chromatin Immunoprecipitation (ChIP)**

ChIP was performed as previously described (27) with modifications. For ChIP following addition of a drug, drug was added 2 hours after cells were transfected with the I-SceI on June 27, 2021. © 2021 American Association for Cancer Research. mct.aacrjournals.org Downloaded from mct.aacrjournals.org on June 27, 2021. © 2021 American Association for Cancer Research.
expression plasmid as described above for the DR-GFP reporter HR assay, and cells were collected 18 hours after transfection. For ChIP following siRNA transfections, cells were transfected with siRNA, incubated 24 hours, and co-transfected with siRNA and I-SceI expression plasmid (as described above for the DR-GFP reporter HR assay). Cells were collected 18 hours after the second transfection. RAD51 was immunoprecipitated using rabbit polyclonal RAD51 ChIP grade antibody (1 µg, Cat. No. ab176458, Abcam) and H3K9me3 was immunoprecipitated using rabbit polyclonal H3K9me3 ChIP grade antibody (1 µg, Cat. No. ab8898, Abcam). The primers that amplify a region 180 base pairs away from the I-SceI cut site in the genomically integrated DR-GFP are listed in Supplementary Table S2.

**EdU (5-ethynyl-2’-deoxyuridine) Staining and Cell Cycle Analysis**

For EdU staining, EdU was added concomitantly with either VLX600 or hydroxyurea to OVCAR-8 cells, and the cells were incubated for 1 hour, fixed and processed per the supplier’s protocol (Cat. No. C10646, Thermo Fisher Scientific), and analyzed by flow microfluorimetry. Bivariate analysis of DNA content (X-axis) and EdU incorporation (Y-axis) was carried out with FlowJo software (BD Biosciences, San Jose, CA). For cell cycle analysis, OVCAR-8 cells (75,000 per well) were plated in 6-well plates, cultured for 16 hours, treated continuously with VLX600 for 72 hours, fixed, and analyzed for DNA content as previously described with FlowJo software (BD Biosciences, San Jose, CA) (28).

**In vitro KDM Assay**
KDM activity assays were performed using the Succinate-Glo™ JmJc Demethylase/Hydroxylase Assay kit (Cat. No. V9900, Promega, Madison, WI) following the supplier’s instructions with 150 ng purified KDM4A (JMJD2A) (Cat. No. 50123, BPS Bioscience, San Diego, CA) and 10 µM H3K9me3 peptide substrate (Cat. No. AS-64452, Anaspec, Fremont, CA) per reaction using a final concentration of 2.5 µM FeCl₂. Percent KDM4A activity is expressed as a percentage of the control with no inhibitor treatment. α-ketoglutarate (10 µM per reaction), ascorbic acid (100 µM per reaction), and FeCl₂ were from Sigma Aldrich, and 96-well half area white flat bottom polystyrene plates were from Corning (Cat. No. 3693).

Seahorse Assay

OVCAR-8 cells were plated at 8,000 cells/well onto Seahorse 8-well XFp cell culture miniplates (Cat. No. 103025-100, Seahorse Bioscience, Agilent Technologies, Santa Clara, CA) and allowed to grow for 24 hours before being assayed for oxygen consumption rate (OCR) on a Seahorse XFp Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA). One hour prior to the start of the assay, cells were washed and changed to Seahorse XF base assay medium supplemented with 2 mmol/L l-glutamine and 10 mmol/L glucose, adjusted to pH 7.4, and incubated in a 37°C non-CO₂ incubator. OCR was measured over 6 hours under basal conditions and after the addition of VLX600 (40 nM or 5 uM).

Apoptosis Assay

OVCAR-8 cells were seeded at 100,000 cells into 10-cm tissue culture dishes, cultured overnight, and treated with vehicle, VLX600, olaparib, or VLX600 + olaparib for 72 hours. The
percentages of apoptotic cells were determined by Annexin V and propidium iodide staining using an APC Annexin V apoptosis assay kit (Cat. No. 550475; BD Biosciences).
Results

**VLX600 synergizes with PARPis in HR-proficient ovarian cancer cells**

In a small-scale screen to identify small-molecules that synergize with PARPis in HR-proficient ovarian cancer cell lines, we found that VLX600, which has minimal cytotoxicity on its own, synergizes with the PARPis olaparib and veliparib in multiple cell lines (Fig. 1A-C; Supplementary Fig. S1A-C), at least in part by inducing apoptosis (Supplementary Fig. S1D-E). Importantly, this synergy occurs at concentrations of VLX600 (20-40 nM) that are well below the peak plasma concentrations of VLX600 that were observed in mice and humans treated with VLX600 (22).

**VLX600 disrupts HR and synergizes with cisplatin in HR-proficient ovarian cancer cells**

Because defects in HR sensitize cancer cells to PARPis (6), we examined if VLX600 disrupts HR using HR-competent OVCAR-8-DR-GFP cells (23), which contain a stably integrated direct-repeat green fluorescent protein (DR-GFP) construct (29). VLX600 concentrations that sensitized cells to PARPis also disrupted repair of this HR substrate (Fig. 2A), and supplementation with exogenous FeCl$_2$ or FeCl$_3$ reversed VLX600 inhibition of HR (Fig. 2B), indicating that the iron chelating ability of VLX600 is required for HR suppression. Notably, VLX600 inhibited HR at concentrations that did not disrupt cell cycle progression or DNA replication (Supplementary Fig. S2A-D), showing that the effects of VLX600 were not caused by the accumulation of cells in a phase of the cycle that does not support HR (e.g., outside of S and G2) or by blocking DNA synthesis. Additionally, the highest VLX600 concentration (i.e., 40 nM VLX600) that was used in the VLX600 and PARPi combination studies (Fig. 1) did not disrupt
oxygen consumption (Supplementary Fig. S2E), a known effect of higher concentrations of VLX600 (20), thus indicating that the HR defect induced by VLX600 was not due to inhibition of mitochondrial respiration.

To assess whether HR disruption by VLX600 plays a role in its synthetic lethal interaction with PARPis, we examined the synergy between VLX600 and olaparib in BRCA2-mutated HR-deficient PEO1 cells. VLX600 did not further sensitize PEO1 cells to olaparib (Fig. 2C), and synergy between these agents was markedly decreased (Fig. 2D) compared to HR-proficient OVCAR-8, PEO14, and OV90 cells (Fig. 1A-C), suggesting that VLX600’s effect on HR contributes to its synergistic interaction with PARPis.

VLX600 also synergized with cisplatin in multiple cell types (Fig. 2E; Supplementary Fig. S3A and S3B). This observation is consistent with the fact that platinum-induced DNA crosslinks are repaired in part by HR (6). Taken together, these findings indicate that VLX600 disrupts HR via an iron chelation-dependent mechanism and sensitizes ovarian cancer cells to PARPis and cisplatin by inhibiting HR.

**VLX600 perturbs the recruitment of HR repair proteins to double-strand breaks (DSBs)**

HR proceeds through a complex series of reactions that begin with the exonucleolytic processing of DNA breaks to ssDNA ends, followed by the deposition of RPA on the ssDNA and the replacement of RPA with RAD51 on the exposed ssDNA in a process that is facilitated by BRCA1 and BRCA2 (30). To determine the point at which VLX600 blocks this pathway, we first analyzed the effects of 100 nM VLX600, a concentration that reduced but did not completely inhibit HR (Fig. 2A), at several steps in the repair pathway. In HR-proficient OVCAR-8 and PEO14
cells, VLX600 (100 nM) blocked RAD51 foci formation at ionizing radiation (IR)-induced DSBs as effectively as the RAD51 inhibitor RI-1 (31) (Fig. 3A-C; Supplementary Fig. S4A, without affecting RAD51 levels (Fig. 3D). Similarly, VLX600 also reduced RAD51 recruitment to DSBs produced by the nuclease I-SceI in OVCAR-8-DR-GFP cells using RAD51 chromatin immunoprecipitation (ChIP) assays (Fig. 3E).

Analyses of the upstream HR events required for RAD51 foci formation showed that VLX600 did not affect end resection or the accumulation of ssDNA at DSBs as assessed by RPA32 foci (Fig. 3F; Supplementary Fig. S5A) or the formation of BRCA1 foci following IR (Fig. 3G; Supplementary Fig. S5B), respectively. VLX600 at 100 nM also did not affect RPA32 and BRCA1 protein levels (Supplementary Fig. S5C). These results indicate that 100 nM VLX600 does not disrupt these early events in HR repair. In contrast, higher concentrations of VLX600 were able to disrupt the formation of BRCA1 foci (Fig. 3H) without affecting BRCA1 levels (Supplementary Fig. S5D). Taken together, these results indicate that VLX600 selectively disrupts RAD51 foci formation at low concentrations. In contrast, at higher concentrations, VLX600 also disrupts BRCA1 foci formation, suggesting that, depending on the concentration, VLX600 may affect multiple targets that regulate HR.

**VLX600 disrupts HR by targeting KDMs**

KDMs are iron-dependent enzymes that were recently shown to regulate HR at multiple steps (15), including RAD51 recruitment to DSBs (17). Because VLX600 is an iron chelator, we hypothesized that VLX600 might disrupt HR by inhibiting KDMs. To address this question, we performed the following analyses. First, to assess whether KDMs participated in HR in ovarian...
cancer cells, we conducted a small-scale siRNA screen of KDMs that were previously reported to affect HR in other model systems (15). These analyses showed that depletion of several KDMs, confirmed by qRT-PCR and/or Western blotting, modestly increased sensitivity to olaparib (Supplementary Fig. S6A).

Second, because the individual members of a KDM family can have overlapping and redundant functions, we next asked whether simultaneously depleting multiple family members enhanced the HR defect. For this analysis, we focused on the KDM4 family because several members of this family were recently reported to promote HR by regulating chromatin structure and the subsequent recruitment of repair factors, including BRCA1 and RAD51 (32), two phenotypes that we observed with VLX600 (Fig. 3A-E and H). The KDM4 family has 5 members (KDM4A, B, C, D, and E). Due to the low prevalence of KDM4D and KDM4E mRNA transcripts in OVCAR-8 cells, we confined our analyses to the effects of co-depleting KDM4A, KDM4B, and KDM4C. These studies showed that co-depletion reduced HR (Fig. 4A; Supplementary Fig. S6B) and sensitized to olaparib (Fig. 4B; Supplementary Fig. S6C) compared to individually depleting each isoform, thus indicating that all three isoforms play a role in HR in ovarian cancer cells. Consistent with these results, KDM4 co-depletion reduced RAD51 association with DSBs (Fig. 4C; Supplementary Fig. S6D) as well as RAD51 and BRCA1 foci formation after ionizing radiation (Fig. 4D and E; Supplementary Fig. S6E). Taken together, these results show that VLX600 and KDM4 co-depletion induce similar phenotypes.

Third, the finding that VLX600 and KDM4 co-depletion (Fig. 3A-E and H; Fig. 4A-E) have similar effects on the HR pathway supports the possibility that VLX600’s effects could be, in part, due to inhibition of KDM4 family members. Consistent with this possibility, VLX600
increased histone H3 Lys 9 trimethylation (H3K9me3), a substrate for KDM4 family members (Fig. 4F), as well as the accumulation of H3K9me3 modifications at DSBs (Fig. 4G). Moreover, in KDM4 co-depleted cells (Supplementary Fig. S6E), VL600 did not further sensitize the cells to olaparib (Fig. 4H and I), and the synergy between VLX600 and olaparib was reduced (Fig. 4J). Finally, VLX600 inhibited recombinant KDM4A activity when added to an in vitro demethylation reaction (Fig. 4K). Collectively, these findings support the notion that VLX600 inhibits KDM family members and that this inhibition disrupts the HR pathway at various points, thus identifying a mechanistic explanation for its synergy with olaparib (Fig. 4L).

Other iron chelators also inhibit HR

The results to this point show that VLX600 disrupts HR by inhibiting iron-dependent KDMs. To determine if other iron chelators also disrupt HR, we next examined the effect of the iron chelators ciclopirox and deferoxamine (DFO) on HR. Both agents inhibited HR (Fig. 5A and 5C), synergized with olaparib (Fig. 5B and 5D; Supplemental Fig. S7A and S7B), reduced RAD51 recruitment to DSBs (Fig. 5E), and increased global H3K9me3 levels (Fig. 5F). Taken together, these results show that other iron chelators also increase histone H3 methylation, disrupt HR, and synergize with PARPis.
Discussion

Here we show that VLX600, as well as other iron chelators, disrupt HR repair and, correspondingly, sensitize HR-proficient ovarian cancer cells to DNA damaging agents and PARPis. Because emerging evidence indicates that HR is also regulated by Fe^{2+}-dependent KDMs (15), we then explored whether VLX600 disrupted HR by inhibiting KDMs. Indeed, we found that depletion of KDM4 family members phenocopies these effects. Furthermore, we showed that VLX600 inhibits KDMs in cells, as demonstrated by increased histone H3K9 trimethylation (Fig. 4F) in cells, and inhibits KDM4A-catalyzed demethylation of a peptide substrate (Fig. 4K) using purified proteins in vitro. Taken together, these results indicate that VLX600 disrupts HR and sensitizes ovarian cancer cells to agents used to treat HGOSC by inhibiting KDM4 demethylases and likely other KDM families that participate in HR.

KDMs demethylate histones and, to a lesser extent, other substrates to regulate DNA transcription, chromatin structure, DNA replication, and cell cycle progression. Histone methylation is also a key regulator of responses to DNA damage, including HR (15). Multiple studies have shown that the activities of histone methyltransferases as well as multiple KDM isoforms are required to modify chromatin structure at sites of DNA damage by catalyzing modifications that suppress transcription and that ultimately open chromatin to allow repair enzymes access to the site of damage. Indeed, both methylation and demethylation of the same histone residues have been shown to affect HR repair. For example, the histone methyltransferases and KDMs that methylate and demethylate histone H3K9, respectively, are recruited to sites of damage where they alter DNA transcription, H3K9 methylation status, HR repair, and sensitivity to DSB-inducing agents (14). Despite this progress, it not yet clear how
these H3K9 methylation and demethylation events are coordinated to orchestrate the multiple steps that direct DNA damage responses.

Because histone methyltransferases and demethylases play pivotal roles in tumor biology, including DNA repair, multiple efforts to develop selective inhibitors are underway (18,19). However, recent studies suggest that less selective KDM inhibitors may also be therapeutically useful. For example, ciclopirox, an iron chelator that is approved as a topical antifungal agent, was shown to selectively kill N-MYC-overexpressing neuroblastoma cell lines due to its ability to inhibit KDMs (33). Similarly, here we report that the iron chelator VLX600, which was originally identified based on its ability to selectively kill metabolically stressed cells in multicellular tumor spheroids by inhibiting mitochondrial respiration (19) also inhibits KDM family members.

The present studies show that VLX600 and other iron chelators disrupt HR by inhibiting KDM4 family members and possibly other KDM isoforms. However, one concern with iron chelators is their potential lack of specificity due to the many iron-dependent processes in eukaryotic cells. Nonetheless, at the concentrations used here, VLX600 appears to have some specificity. For example, VLX600 did not disrupt the cell cycle, indicating that it did not inhibit ribonucleotide reductase, an iron-dependent enzyme that is required for the production of dNTPs (34). Additionally, VLX600 did not alter the global methylation status of H3K4me3 or H3K36me2 (Supplemental Fig. S6G), thus demonstrating that it does not affect all KDM-dependent demethylations. Finally, the fact that VLX600 did not have unmanageable toxicities in humans or in mice at doses that had antitumor activity in mice further suggests some level of specificity.
These findings also raise the possibility that VLX600 or other iron chelators represent a novel way to pharmacologically induce an HR-deficient state, which is associated with better patient survival in HGSOC with preexisting HR defects (4-6). Interestingly, both ciclopirox and VLX600 have been studied as anticancer agents in mouse models and humans. When administered systemically, ciclopirox had antineoplastic activity in N-MYC-overexpressing neuroblastoma xenografts (35). Oral ciclopirox also demonstrated modest single-agent activity in a phase 1 clinical trial in humans, with peak plasma concentrations of 0.2 – 0.9 µM (36).

Moreover, in a human phase 1 trial of single-agent VLX600 that did not identify a maximum tolerated dose, the best response was stable disease (32%) in solid tumors (22). In this study, VLX600 was administered intravenously on days 1, 8, and 15 of each 28-day treatment cycle. VLX600 reached peak plasma concentrations of >2.5 µM, with an elimination half-life of >9 hours, a mean residence time of ~9 hours, and a volume of distribution ranging from 1.1 – 5.1 L/kg. Taken together, these studies show that iron chelators can be administered to humans at doses that lead to plasma concentrations that far exceed the nanomolar concentrations required to inhibit KDMs and disrupt HR in cell culture experiments. Importantly, however, it is not known whether these agents actually inhibit KDMs and HR under these in vivo conditions.

The present studies have several additional limitations that raise key questions that will be addressed in future preclinical studies combining VLX600 (or ciclopirox) with carboplatin or PARP inhibitor in HGSOC patient-derived xenografts in mice. First, it is not known whether VLX600 combination therapies, compared to each agent singly, are more efficacious in mouse models of HGSOC, nor is it known whether inhibiting HR correspondingly increases toxicities in normal tissues that are caused by genotoxic chemotherapies. However, two lines of evidence
suggest that there might be a therapeutic window. VLX600 plus oxaliplatin was more effective than oxaliplatin alone without substantial systemic toxicity in xenograft models of colon cancer (20). Additionally, three early phase 1 clinical trials were recently reported with berzosertib, an inhibitor of the checkpoint kinase ATR, which positively regulates HR and is activated by replication stress and DNA damage (23,37). In these trials, berzosertib was combined with carboplatin (38), topotecan (39), or gemcitabine (40). Critically, these trials demonstrated that berzosertib inhibited ATR inhibition, had antitumor activity, and had acceptable toxicity, thus suggesting that it is possible to pharmacologically inhibit fundamental cellular DNA damage responses in humans treated with genotoxic chemotherapies.

Second, even though pharmacokinetic studies in humans showed that plasma levels of VLX600 and ciclopirox were higher than required to disrupt HR in cell lines, additional studies will need to address whether KDMs and HR are actually inhibited in tumors by VLX600 in mouse models.

Third, despite the fact that KDMs that regulate HR are inhibited by very low concentrations of VLX600, the observation that iron chelators have multiple targets, raises the possibility that these additional pleiotropic effects could either contribute to or counteract the activity of genotoxic chemotherapies.

Finally, it will be essential to define the appropriate dose-timing strategies of VLX600 with respect to chemotherapy administration. In the phase 1 trial of VLX600 (22), the agent was given weekly as an intravenous infusion. Additional studies are needed to determine if antitumor activity is greatest if VLX600 is given before, during, or after platinum compounds, which are also typically dosed intermittently by intravenous infusion. Additionally, because
PARP inhibitors are typically dosed on a daily basis orally, it will be necessary to determine if the intermittent dosing regimen used in the phase 1 trial of VLX600 will augment the activity of daily PARPi dosing in mouse PDX models.

In summary, our findings demonstrate that VLX600 and other iron chelators inhibit KDMs that regulate HR repair, thereby disabling HR and enhancing the cytotoxicities of PARPi and platinum agents. Our findings also are the first to provide the mechanistic basis for the previously observed synergy between VLX600 and platinum agents in colon cancer cells (19). Finally, our results suggest a potential way to pharmacologically inhibit HR using iron chelators to augment the activity of platinum compounds and PARP inhibitors, two standards-of-care for HGSOC.

Acknowledgements

The authors acknowledge Yuichi Machida, Ph.D., Martin Fernandez-Zapico, M.D., and Catherine Huntoon for technical assistance, and Kyle Howell of the Mayo Clinic Microscopy and Cell Analysis Core. This work was supported by NIH (R01 CA194498 to L.M.K.), a Mayo Clinic Ovarian Cancer SPORE Career Enhancement Award (P50 CA136393 to A.K.), a Foundation for Women’s Cancer Genentech Ovarian Cancer Young Investigator Career Development Award (to A.K.), a Wallace and Evelyn Simmers Career Development Award for Ovarian Cancer Research (to A.K.), and an Ovarian Cancer Research Alliance Liz Tilberis Early Career Award (to A.K.).
References

10. Pascal JM. The comings and goings of PARP-1 in response to DNA damage. DNA Repair (Amst) 2018;71:177-82


Figure Legends

Figure 1. VLX600 synergizes with olaparib in ovarian cancer cells. A-C, OVCAR-8 (A), PEO14 (B), and OV90 (C) cells were plated; allowed to adhere for 4 hours; treated with indicated concentrations of vehicle, olaparib, or VLX600; and cultured in the continued presence of these agents for 8 to 14 days to allow colony formation (left). Colonies were stained and manually counted. Data are normalized to vehicle-only treated controls. Left-hand and middle panels show the mean ± SEM of three technical replicates from a single experiment that is representative of three independent experiments. Combination index (CI) values (right-hand panels) were calculated using the Chou-Talalay method in Calcusyn. CI > 1, CI = 1, and CI < 1 are antagonistic, additive, and synergistic, respectively. Right-hand panels show the mean ± SEM of three independent experiments.

Figure 2. VLX600 disrupts HR repair, has limited effects in HR-deficient cells, and synergizes with cisplatin. A, OVCAR-8-DR-GFP cells were transfected with I-SceI expression plasmid, plated, incubated 2 hours, treated with vehicle or indicated concentrations of VLX600, cultured in the continued presence of VLX600 for 72 hours, and analyzed for GFP expression by flow cytometry. GFP-positive (GFP+) cells are expressed as a percentage of vehicle-treated controls. Mean ± SEM of three independent experiments. B, OVCAR-8 cells were transfected and treated as in (A) but with the addition of 10 µM FeCl₂ or FeCl₃ at the time of VLX600 addition. Mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, unpaired Student’s t-test. C-D, the effects of olaparib and VLX600 in PEO1 cells were assessed using colony-forming assays as described in Fig. 1. (C) shows the mean ± SEM of three technical replicates from a single
experiment that is representative of three independent experiments; (D) shows the mean ± SEM of three independent experiments. E, the effects of cisplatin and VLX600 on OVCAR-8 cells were assessed using colony-forming assays as in Fig. 1. Left panel shows the mean ± SEM of three technical replicates from a single experiment that is representative of three independent experiments; Right panel shows the mean ± SEM of three independent experiments. Combination index (CI) values (right) were calculated as in Fig. 1. Error bars, means ± SEM of three independent experiments.

**Figure 3. VLX600 reduces recruitment of HR proteins to sites of DNA damage.** A-D, OVCAR-8 (A, B) and PEO1 cells (C) were pre-treated with 100 nM VLX600 or 50 µM RAD51 inhibitor RI-1 for 2 hours, exposed to 2 Gy ionizing radiation, incubated for 6 hours, and then co-immunostained for RAD51 and γ-H2AX (A-C) and immunoblotted for RAD51 (D). β-actin served as the loading control. Representative 100X images foci are shown in (A). Scale bars, 10 µm. Co-localized foci were manually counted. (B, C), Mean ± SEM of three independent experiments. E, OVCAR-8-DR-GFP cells were transfected with an I-SceI expression plasmid. Two hours after plating, VLX600 was added, cells were cultured for 16 hours, and then processed for ChIP using RAD51 and control immunoglobin G (IgG) antibodies and qRT-PCR using primers that amplified a region 180 base pairs away from the I-SceI-induced DSB. Mean ± SEM of four independent experiments. F-G, OVCAR-8 cells were pre-treated with 100 nM VLX600 for 2 hours, exposed to 2 Gy ionizing radiation, incubated for 4 or 6 hours, respectively, co-immunostained for RPA32 or BRCA1 and γ-H2AX and co-localized foci were manually counted. Mean ± SEM of three independent experiments. H, OVCAR-8 cells were pre-treated with indicated concentrations of
VLX600 for 2 hours, exposed to 2 Gy ionizing radiation, incubated for 6 hours, and co-immunostained for BRCA1 and γ-H2AX. Co-localized foci were manually counted. Mean ± SEM of three independent experiments. ns, not significant, *p < 0.05, **p < 0.01, ****p < 0.0001, unpaired Student’s t-test.

Figure 4. VLX600 reduces HR by inhibiting KDM histone demethylases. A, OVCAR-8-DR-GFP cells were transfected with individual siRNAs (negative control siLuc, positive control siBRCA1, or independent siRNAs targeting KDM4A [A#1, A#2], KDM4B [B#1, B#2], and KDM4C [C#1, C#2]), or co-transfected with a mixture of siRNAs targeting all three KDM4 family members (A#2, B#1, C#1). After 24 hours, cells were co-transfected with I-SceI plasmid and the same siRNAs as used in the first transfection, and incubated for 72 hours. Cells were then analyzed by immunoblotting (Supplementary Figure S6B) and for GFP expression by flow cytometry. GFP expression is expressed as a percentage of the siLuc siRNA control. Mean ± SEM of at least three independent experiments. B, OVCAR-8 cells were transfected twice (24 hours between transfections) with individual siRNAs (negative control siLuc, positive control siBRCA1, siKDM4A #2, siKDM4B #1, siKDM4C #1) or were co-transfected with a mixture of KDM4 siRNAs (siKDM4A #2, siKDM4B #1, siKDM4C #1) and cultured for 48 hours. Cells were then trypsinized and immunoblotted (Supplementary Figure S6C) or re-plated for clonogenic assay. Four hours after re-plating, cells were treated with olaparib and cultured for 8 to 10 days to allow colony formation. Colonies were manually counted. Mean ± SEM of three technical replicates from a single representative experiment of three independent experiments. C, OVCAR-8-DR-GFP cells were transfected with negative control siLuc, positive control siBRCA1, or a mixture of three
KDM4 family member siRNAs (siKDM4A #2, siKDM4B #1, siKDM4C #1). After 24 hours, cells were co-transfected with I-SceI expression plasmid and the same siRNA as used in the first transfection, incubated for 18 hours, and analyzed by immunoblotting (Supplementary Figure S6D) and by ChIP as in Fig. 3E. Mean ± SEM of three independent experiments. D and E, OVCAR-8 cells were transfected with negative control siLuc siRNA or co-transfected with a mixture of KDM4 siRNAs (siKDM4A #2, siKDM4B #1, siKDM4C #1) and cultured for 48 hours. Cells were then exposed to 2 Gy ionizing radiation, incubated for 6 hours, and then co-immunostained for RAD51 and γ-H2AX (D) or BRCA1 γ-H2AX (E). Co-localized foci were manually counted (B, C). Mean ± SEM of three independent experiments. F, OVCAR-8 and PEO14 cells were treated with 100 nM or 150 nM VLX600 continuously for 24 hours and histone extracts were immunoblotted for H3K9me3 and total histone H3. Immunoblot shown is representative of three independent experiments. F, OVCAR-8 and PEO14 cells were treated with 100 nM or 150 nM VLX600 for 24 hours and histone extracts were immunoblotted for H3K9me3 and total histone H3. Immunoblot shown is representative of three independent experiments. G, VLX600 increases H3K9me3 at I-SceI-induced DSBs. OVCAR-8-DR-GFP cells were transfected with an I-SceI expression plasmid. Two hours after plating, VLX600 was added, cells were cultured for 16 hours and then processed for ChIP as in Fig. 3E except that H3K9me3 antibody was used in place of RAD51 antibody. H-J, OVCAR-8 cells were transfected with negative control siLuc siRNA (H) or co-transfected with a mixture of KDM4 siRNAs (siKDM4A #2, siKDM4B #1, siKDM4C #1) (I) and cultured for 48 hours. Cells were then trypsinized and immunoblotted (Supplementary Figure 6E) and subjected to colony-forming assays. For colony-forming assays, cells were plated, incubated 4 hours, and treated with indicated concentrations of olaparib with or without 40 nM
VLX600, and cultured for 8 to 10 days to allow colony formation. Colonies were manually counted. Mean ± SEM of three technical replicates from a single representative experiment of three independent experiments. (J) shows combination index (CI) values calculated using the Chou-Talalay method in CalcuSyn. Error bars, means ± SEM of three independent experiments. K, KDM4A was incubated with H3K9me3 peptide substrate using a succinate detection luminescent assay (IC50, approx. 3.7 µM). Mean ± SEM of three independent experiments. L, Proposed model of HR disruption by VLX600. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired Student’s t-test.

Figure 5. Ciclopirox and deferoxamine induce histone H3 methylation, disrupt HR, and synergize with olaparib. A and C, OVCAR-8-DR-GFP cells were transfected, treated with ciclopirox or deferoxamine (DFO), and analyzed for GFP as described in Fig. 2A. Mean ± SEM of three independent experiments. B and D, Clonogenic assays were performed as described in Fig. 1. Graphs show mean ± SEM of three technical replicates from a single experiment that is representative of three independent experiments. E, OVCAR-8-DR-GFP cells were transfected with an I-SceI expression plasmid. Two hours after plating, ciclopirox or deferoxamine was added, and cells were cultured for 16 hours and processed for ChIP analyses as in Fig. 3E. Mean ± SEM of four and three independent experiments for ciclopirox and deferoxamine, respectively. F, OVCAR-8 cells were treated with ciclopirox or deferoxamine continuously for 24 hours, and histone extracts were immunoblotted for H3K9me3 and total histone H3. Image shows a representative experiment of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired Student’s t-test.
**Figure 1**

(A) **OV CAR-8**

- Colonies (% Control) vs. VLX600 (nM)
- Combination Index vs. Fraction Affected

(B) **PEO14**

- Colonies (% Control) vs. VLX600 (nM)
- Combination Index vs. Fraction Affected

(C) **OV90**

- Colonies (% Control) vs. VLX600 (nM)
- Combination Index vs. Fraction Affected

**Legend:**
- Olaparib alone
- Olaparib + VLX600 (30 nM)
- Olaparib + VLX600 (40 nM)

**Combination Index:**
- Antagonism
- Synergism
**Figure 2**

A  
**OVCA8-DR-GFP**

B

**OVCA8-DR-GFP**

C

**PEO1**

D

**PEO1**

E

**OVCA8**

**Olaparib + VLX600 (40 nM)**

**Olaparib + VLX600 (50 nM)**

**Colonies, (% Control)**

**Combination Index**

**Fraction Affected**

**VLX600 (nM) 0 25 50 75 100**

**OVCAR-8**

**Colonies, (% Control)**

**VLX600 (nM) 0 30 40**

**Olaparib (+) - - - + + - +**

**Fe+ (10 µM) - - + - - - -**

**Fe2+ (10 µM) - - + - - - -**
Figure 3  

**A**  

**OVCAR-8**  

After 6 h IR (2 Gy)  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>VLX600 100 nM</th>
<th>RI-1 50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>γ-H2Ax</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Merge + DNA</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**  

**OVCAR-8**  

Cells with ≥5 RAD51 and γ-H2Ax co-localized foci (%)  

<table>
<thead>
<tr>
<th>VLX600 (nM)</th>
<th>0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLX600 100 nM</td>
<td><strong>20</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>

**C**  

**PEO14**  

Cells with ≥5 RAD51 and γ-H2Ax co-localized foci (%)  

<table>
<thead>
<tr>
<th>VLX600 (nM)</th>
<th>0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLX600 100 nM</td>
<td><strong>20</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>

**D**  

**OVCAR-8**  

24 h  

VLX600 (nM) | 0 | 100 kDa |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
</tr>
<tr>
<td>β-actin</td>
<td><img src="image12" alt="Image" /></td>
<td><img src="image13" alt="Image" /></td>
</tr>
</tbody>
</table>

**E**  

**OVCAR-8-DR-GFP**  

% Input, (% Control)  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>VLX600 100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>IgG</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
</tr>
</tbody>
</table>

**F**  

**OVCAR-8**  

Cells with ≥5 RPA32 and γ-H2Ax co-localized foci (%)  

<table>
<thead>
<tr>
<th>VLX600 (nM)</th>
<th>0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLX600 100 nM</td>
<td><img src="image18" alt="Image" /></td>
<td><img src="image19" alt="Image" /></td>
</tr>
</tbody>
</table>

**G**  

**OVCAR-8**  

Cells with ≥5 BRCA1 and γ-H2Ax co-localized foci (%)  

<table>
<thead>
<tr>
<th>VLX600 (nM)</th>
<th>0</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLX600 100 nM</td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
</tr>
</tbody>
</table>

**H**  

**OVCAR-8**  

Cells with ≥5 BRCA1 and γ-H2Ax co-localized foci (%)  

<table>
<thead>
<tr>
<th>VLX600 (nM)</th>
<th>0</th>
<th>150</th>
<th>200</th>
<th>300</th>
<th>400</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLX600 150 nM</td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
</tr>
<tr>
<td>VLX600 200 nM</td>
<td><img src="image28" alt="Image" /></td>
<td><img src="image29" alt="Image" /></td>
<td><img src="image30" alt="Image" /></td>
<td><img src="image31" alt="Image" /></td>
<td><img src="image32" alt="Image" /></td>
<td><img src="image33" alt="Image" /></td>
</tr>
<tr>
<td>VLX600 300 nM</td>
<td><img src="image34" alt="Image" /></td>
<td><img src="image35" alt="Image" /></td>
<td><img src="image36" alt="Image" /></td>
<td><img src="image37" alt="Image" /></td>
<td><img src="image38" alt="Image" /></td>
<td><img src="image39" alt="Image" /></td>
</tr>
<tr>
<td>VLX600 400 nM</td>
<td><img src="image40" alt="Image" /></td>
<td><img src="image41" alt="Image" /></td>
<td><img src="image42" alt="Image" /></td>
<td><img src="image43" alt="Image" /></td>
<td><img src="image44" alt="Image" /></td>
<td><img src="image45" alt="Image" /></td>
</tr>
<tr>
<td>VLX600 500 nM</td>
<td><img src="image46" alt="Image" /></td>
<td><img src="image47" alt="Image" /></td>
<td><img src="image48" alt="Image" /></td>
<td><img src="image49" alt="Image" /></td>
<td><img src="image50" alt="Image" /></td>
<td><img src="image51" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 4

A. OVCAR-8-DR-GFP

B. OVCAR-8

C. OVCAR-8-DR-GFP

D. OVCAR-8

E. OVCAR-8

F. OVCAR-8

G. OVCAR-8-DR-GFP

H. OVCAR-8 siLuc

I. OVCAR-8 siKDM4A+B+C

J. OVCAR-8

K. KDM4A Activity

L. HR-proficient HGSOC cells

Combination Index

Fraction Affected

% KDM4A Activity

IC50 = ~3.7 µM

Increased sensitivity to platinum agents and PARPis

Downloaded from mct.aacrjournals.org on June 27, 2021. © 2021 American Association for Cancer Research.
Molecular Cancer Therapeutics

VLX600 Disrupts Homologous Recombination and Synergizes with PARP Inhibitors and Cisplatin by Inhibiting Histone Lysine Demethylases

Thomas L. Ekstrom, Nicholas M. Pathoulas, Amelia M. Huehls, et al.

Mol Cancer Ther Published OnlineFirst June 17, 2021.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-20-1099

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2021/06/02/1535-7163.MCT-20-1099.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2021/06/16/1535-7163.MCT-20-1099. Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.