Poly(ADP-Ribose) Polymerase Inhibition Synergizes with 5-Fluorodeoxyuridine but not 5-Fluorouracil in Ovarian Cancer Cells

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Abstract

5-Fluorouracil (5-FU) and 5-fluorodeoxyuridine (FdUrd, floxuridine) have activity in multiple tumors, and both agents undergo intracellular processing to active metabolites that disrupt RNA and DNA metabolism. These agents cause imbalances in deoxynucleotide triphosphate levels and the accumulation of uracil and 5-FU in the genome, events that activate the ATR- and ATM-dependent checkpoint signaling pathways and the base excision repair (BER) pathway. Here, we assessed which DNA damage response and repair processes influence 5-FU and FdUrd toxicity in ovarian cancer cells. These studies revealed that disabling the ATM, ATR, or BER pathways using small inhibitory RNAs did not affect 5-FU cytotoxicity. In stark contrast, ATR and a functional BER pathway protected FdUrd-treated cells. Consistent with a role for the BER pathway, the poly(ADP-ribose) polymerase (PARP) inhibitors ABT-888 (veliparib) and AZD2281 (olaparib) markedly synergized with FdUrd but not with 5-FU in ovarian cancer cell lines. Furthermore, ABT-888 synergized with FdUrd far more effectively than other agents commonly used to treat ovarian cancer. These findings underscore differences in the cytotoxic mechanisms of 5-FU and FdUrd and suggest that combining FdUrd and PARP inhibitors may be an innovative therapeutic strategy for ovarian tumors. Cancer Res; 71(14); 1–11. ©2011 AACR.

Introduction

5-Fluorouracil (5-FU) has activity in multiple neoplastic diseases and is one of the most widely used chemotherapy agents. 5-FU enters cells by facilitated transport and undergoes extensive metabolism to multiple active metabolites (Fig. 1A, review in [1]). On the one hand, 5-FU can be converted to the ribonucleotide FUTP (5-fluorouridine triphosphate), which exerts cytotoxic activity when it is incorporated into RNAs by RNA polymerases. On the other hand, 5-FU also has complex effects on DNA replication following its conversion to the active metabolites FdUMP [5-fluorodeoxyuridine (FdUrd) monophosphate] and FdUTP (5-FdUrd triphosphate). Whereas FdUTP is incorporated directly into DNA, FdUMP inhibits thymidylate synthase, resulting in depletion of dTTP, accumulation of dUTP and its subsequent incorporation into DNA, and disruption of deoxynucleotide triphosphate (dNTP) ratios.

In addition to being a metabolite of 5-FU, FdUrd (also known as floxuridine) is an FDA-approved drug for the treatment of hepatic colon metastases (2). Moreover, the drug has activity in multiple cancers, including ovarian cancer (3–11). Unlike 5-FU, however, FdUrd is generally believed to exert its antiproliferative effects primarily through the disruption of DNA replication (i.e., by inhibiting thymidylate synthase and/or causing the incorporation of 5-FU into genomic DNA; ref. 12). Thus, in addition to being a useful clinical agent, FdUrd is also frequently used by basic researchers as a means to specifically focus on 5-FU’s DNA-directed effects.

Nucleoside analogs, including 5-FU and FdUrd, disrupt dNTP levels and are incorporated into DNA, 2 events that stall DNA replication and activate ATR (13–22), an apical kinase in the ATR checkpoint signaling pathway. Activated ATR phosphorylates multiple substrates, including the kinase Chk1. Collectively, ATR and Chk1 phosphorylate substrates that promote cell survival by impeding cell-cycle progression, orchestrating DNA repair and stabilizing stalled replication forks (23). Notably, however, FdUrd and 5-FU also induce double-stranded DNA breaks (24, 25), which activate the ATM signaling pathway (26), including the ATM substrate checkpoint kinase 2 (Chk2). Like the ATR pathway, the ATM pathway promotes survival of cells with double-stranded DNA breaks by blocking cell-cycle progression and mobilizing DNA repair machinery. Although both the ATR and ATM signaling pathways are activated by 5-FU and FdUrd, the roles these
pathways play in regulating the survival of human tumors treated with these agents have not been explored fully.

The genomically incorporated uracil (U) and 5-FU are also targets of the base excision repair (BER) machinery (12). In this repair pathway, nonbulky DNA lesions are first recognized and cleaved by a DNA glycosylase, producing an abasic site, which is further processed to a single-stranded DNA break by an endonuclease activity such as apurinic/apyrimidinic endonuclease 1 (27). The single-stranded DNA break attracts poly(ADP-ribose) polymerase 1 or 2 (collectively referred to as PARP), which subsequently poly(ADP-ribosyl)ates itself and other proteins, leading to the binding of the scaffolding protein XRCC1 and additional proteins required for completion of BER (28).

Despite our in-depth understanding of the BER machinery, surprisingly little is known about how U and 5-FU DNA lesions are processed in tumor cells treated with 5-FU or FdUrd. Although there are 4 known uracil glycosylases—UNG, SMUG1, TDG, and MBD4—that can excise these lesions in vitro, it remains unclear what roles these glycosylases play in human tumor cells (12). Similarly, the roles of the downstream repair proteins remain poorly explored in human cells, particularly the role of PARP. Given that small molecule PARP inhibitors are now in clinical development as single agents for the treatment of tumors with defects in BRCA1-/BRCA2-dependent repair or as sensitizers to other chemotherapy agents (28), this question may be relevant for the development of novel therapies that include PARP inhibitors.

Here, we have systematically explored the checkpoint and DNA repair processes that are important in ovarian cancer cells treated with 5-FU and FdUrd. Our studies show that 5-FU and FdUrd have distinct mechanisms of action in these tumor cells. Based on these findings, we have discovered that small molecule PARP inhibitors synergize with FdUrd but not with 5-FU, raising the possibility that a combination of FdUrd and a PARP inhibitor may have activity in ovarian cancer.

Materials and Methods

Cell lines and culture

A2780, OVCAR-3, OVCAR-5, OVCAR-8, and SKOV3.ip cells were cultured at 37°C in 5% CO2 with 10% fetal bovine serum (Atlanta Biologicals) in the following media: A2780 and OVCAR-3, RPMI-1640 supplemented with 10 μg/mL insulin (Gibco); OVCAR-5 and OVCAR-8, RPMI-1640 (Mediatech); SKOV3.ip, Dulbecco’s modified Eagle’s medium (Mediatech);

Figure 1. 5-FU and FdUrd activate ATM and ATR checkpoints and induce DNA damage in ovarian cancer cells. A, intracellular metabolism of 5-FU and FdUrd. B and C, OVCAR-3 and SKOV3ip cells were treated with 300 μmol/L 5-FU, 300 μmol/L FdUrd, or 10 mmol/L HU for the indicated times. Cell extracts were blotted for P-Ser317-Chk1 (P-Chk1), P-Thr68-Chk2 (P-Chk2), Chk1, and Chk2. D, SKOV3.ip and OVCAR-8 cells were treated with 300 μmol/L 5-FU, 300 μmol/L FdUrd for 24 hours, or 10 mmol/L HU for 8 hours, fixed, and stained with anti-P-Ser139-histone H2AX (red) and Hoechst 33342 to detect DNA (blue). TS, thymidylate synthase; TP, thymidine phosphorylase; UP, uridine phosphorylase; UK, uridine kinase; OPRT, orotate phosphoribosyltransferase; RR, ribonucleotide reductase; FUR, 5-fluorouridine; FUMP, 5-fluorouridine monophosphate; FUDP, 5-fluorouridine diphosphate; FdUTP, 5-FdUrd monophosphate; FdUDP, 5-FdUrd diphosphate; FdUTP, 5-FdUrd triphosphate.
and WS1, MEM (Mediatech), OSEtsT/human telomerase reverse transcriptase (hTERT) cells (29) were cultured in 5% CO\textsubscript{2} at 34°C in a 1:1 mixture of M199/MCD-105 (Sigma) supplemented with 10% fetal bovine serum and 20 μg/mL hygromycin B. For clonogenic assays, the aforementioned media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech). OVCAR-3 and WS1 cell lines were obtained from American Type Culture Collection, which authenticated the lines by short tandem repeat profiling. OVCAR-5 and OVCA-8 were gifts from D. Scudierio [National Cancer Institute (NCI)]. SKOV3ip, A2780, and OSEtsT/hTERT cells were gifts from V. Shridhar (Mayo Clinic, Rochester, MN) and were genotyped shortly before acquisition. Every 3 months, all cell lines were reintroduced from cryopreserved stocks prepared immediately after receipt from the indicated sources.

Materials
Reagents were from the following suppliers: 5-FU (APP Pharmaceuticals), FdUrd (Bedford Laboratories), ABT-888 (Selleck Chemicals and ChemieTek), AZD2281 (ChemieTek), cisplatin (Teva Pharmaceuticals), gemcitabine (Eli Lilly), oxaliplatin (Tocris), carboplatin (NovoPlus), temozolomide (Cancer Therapy Evaluation Program, NCI), SuperSignal Pico West (Thermo Scientific), and Annexin V FITC and Annexin V Binding Buffer (BD Pharmingen). Reagents for MTS assays were obtained from Sigma-Aldrich.

Antibodies to the following antigens were as follows: phospho (P)-Ser317-Chk1 (R&D Systems); P-Thr68-Chk2, ATR, thymidylate synthase, horseradish peroxidase-linked rabbit immunoglobulin (Ig) G (IgG), and horseradish peroxidase-linked mouse IgG (Cell Signaling); Chk1 (Santa Cruz Biotechnology); Chk2 and ATM (Epitomics); P-Ser139-H2AX (Millipore); XRCC1 (Bethyl Laboratories); fluorescein-conjugated goat anti-mouse IgG (Invitrogen); PARP1, G.G. Poirier (Université Laval, Sillery, QC, Canada); β-actin (Sigma-Aldrich); and HSP90, D. Toft (Mayo Clinic).

Cell transfections and siRNAs
siRNAs (400 nmol/transfection) were mixed with 5 × 10\textsuperscript{6} to 1 × 10\textsuperscript{7} cells in 0.2 mL RPMI-1640 containing 10% fetal bovine serum in a 0.4-cm electroporation cuvette and electroporated with two 10-μs, 280-V pulses in a BTX T820 square wave electroporator (Harvard Apparatus). The transfected cells were cultured for 48 hours before use. Sequences of siRNAs were: ATM-1, 5′-AAGCACCAGTCCGATTGCC-3′ (30); ATR-2, 5′-CCCTCGTGATGGTCGCTGA-3′ (31); XRCC1-2, 5′-CCUGACUCAGUGCAAGAU-3′ (32); XRCC1-3, 5′-CCAGGAA-GATATAGACATT-3′; PARP1-1, 5′-AAGCCUCGCGCCUGUAA-3′ and PARP1-2, 5′-AAGAUAGAGCGUGAAGGCCA-3′; and luciferase, 5′-CTTACGCUGUACUCUCCA-3′ (34).

Cell-cycle analyses, clonogenic assays, cell lysis, immunostaining, Annexin V staining, MTS assays, and cell irradiation
Cell-cycle analyses, clonogenic assays, cell lysis, immunoblotting, and immunostaining were done as described (35, 36). For clonogenic assays using nontransfected cells, percent survivals of all individual and combination treatments were normalized to cells treated with vehicle only. For clonogenic assays using cells transfected with siRNA, percent survivals at each drug concentration were normalized to the vehicle-treated control for the given siRNA. For MTS assays, 2,000 to 3,000 log-phase OSEtsT/hTERT or WS1 cells were plated in 96-well plates, and after 4 hours, the indicated concentrations of ABT-888 and FdUrd were added. The plates were incubated for 4 days and reacted with MTS and phenazine methosulfate as instructed by the supplier for 2 to 3 hours, and absorbances at 490 nm were determined. All percent cell viabilities were normalized to controls treated with vehicle only. Annexin V staining was done according to supplier’s protocols. Cells were irradiated with a RS-2000 Biological Irradiator, Rad Source, 4 to 6 hours after plating.

Results

5-FU and FdUrd activate checkpoint kinases
Previously published results have shown that antimetabolites, including 5-FU and FdUrd, activate checkpoint signaling pathways (15–22, 37). Accordingly, we found that 5-FU and FdUrd induced phosphorylation of Chk1 and Chk2 in 2 ovarian cancer cell lines, OVCAR-8 (Fig. 1B) and SKOV3ip (Fig. 1C). In the SKOV3ip cells, 5-FU induced Chk1 and Chk2 activation in 8 and 24 hours, with levels similar to those seen with the ribonucleotide reductase inhibitor hydroxyurea (HU), which was used as a positive control. In contrast, FdUrd triggered modest and delayed Chk1 phosphorylation in these cells. In OVCAR-8 cells, 5-FU induced delayed Chk1 and Chk2 activation, whereas FdUrd caused rapid Chk1 phosphorylation. Consistent with the observed effects on checkpoint signaling, 5-FU and FdUrd induced phosphorylation of histone H2AX (Fig. 1D), a marker of DNA damage (38). Taken together, these results show that both fluoropyrimidines induce DNA damage and activate the ATM and ATR checkpoint signaling pathways.

ATR but not ATM is important for FdUrd toxicity
Activation of Chk1 and Chk2 suggests that signaling through ATM and/or ATR, both of which affect the survival of cells treated with multiple distinct genotoxins, may influence the toxicity of these agents. To assess how these kinases impact 5-FU and FdUrd cytotoxicity, OVCAR-8 cells were transfected with siRNAs that deplete ATM (ATM-1) and ATR (ATR-2). These siRNAs showed no cytotoxicity on their own (ATM siRNA–transfected cell plating efficiency = 106.7 ± 4.3%, mean ± SEM, n = 4; ATR siRNA–transfected cell plating efficiency = 100.3 ± 11.8%, n = 5, compared with luciferase siRNA-transfected cells) and did not affect formation of the FdUMP-thymidylate synthase complex, indicating that they did not alter uptake and/or metabolism of 5-FU and FdUrd (Supplementary Fig. S1A). Surprisingly, neither ATM nor ATR depletion sensitized either cell line to 5-FU (Fig. 2A and B, left), showing that even though the ATM and ATR pathways are activated, they do not protect these cell lines from 5-FU. Far different results were seen with FdUrd. Whereas ATM
Depletion (which sensitized to ionizing radiation, Supplementary Fig. S1B) had no effect on FdUrd cytotoxicity, ATR depletion profoundly sensitized the cells to FdUrd (Fig. 2A and B, right; see Supplementary Fig. S2A for replotted data highlighting the effects of low concentrations of FdUrd). Furthermore, cells simultaneously depleted by ATM and ATR were not more sensitive to FdUrd than cells depleted only of ATR, indicating that even when ATR levels are severely reduced, ATM does not affect FdUrd toxicity.

Depletion of XRCC1 and PARP1 enhances sensitivity to FdUrd but not 5-FU

The results presented in Figure 2 indicated that disabling checkpoint signaling affected the cytotoxicity of FdUrd but not 5-FU, increasing the possibility that these agents may also differ in their requirements for DNA repair. Because genomically incorporated U and 5-FU are substrates for the BER pathway, we investigated the role of BER in the cytotoxicity of these agents by depleting OVCAR-8 cells of XRCC1, a BER scaffolding protein. siRNA-mediated reduction of XRCC1 moderately reduced plating efficiency (XRCC1-2 = 71.2 ± 5.3, mean ± SEM, n = 4; XRCC1-3 = 81.2 ± 10.3, n = 3 compared with luciferase siRNA–transfected cells). Notably, however, even after adjusting for the reduced plating efficiency, XRCC1 depletion with 2 different siRNAs sensitized to FdUrd, whereas it did not affect 5-FU cytotoxicity (Fig. 3A; see Supplementary Fig. S2B for alternate X-axis scale in Fig. 3A; and Supplementary Fig. S2C for additional XRCC1 siRNA). Similarly, depletion of PARP1 (which did not affect plating efficiency; PARP1-1 = 102.7 ± 2.5, mean ± SEM, n = 3; PARP1-2 = 101.6 ± 9.2, n = 3, compared with luciferase–transfected cells) did not sensitize to 5-FU but did sensitize to FdUrd (Fig. 3B; see Supplementary Fig. S2B for alternate X-axis scale in Fig. 3B). These data indicate that BER is important in ovarian cancer cells treated with FdUrd but not 5-FU. Furthermore, taken in conjunction with our checkpoint signaling studies (Fig. 2), they are consistent with the idea that 5-FU does not kill these cells by inflicting DNA damage.
Small-molecule PARP inhibitors potentiate the antiproliferative activity of FdUrd but not 5-FU

Given that PARP1 depletion increased the cytotoxicity of FdUrd, we reasoned that small-molecule inhibitors of PARP would affect the sensitivity of ovarian cancer cells to this agent. Continuous exposure to either AZD2281 (olaparib) or ABT-888 (veliparib), 2 PARP inhibitors currently in clinical trials, had minimal effects on the cloning efficiency as single agents, with 3 μmol/L ABT-888 reducing the surviving fraction to 90.3 ± 2.4% (mean ± SEM, n = 12). In contrast, both PARP inhibitors markedly increased killing when cells were coexposed to FdUrd and the PARP inhibitor for 24 hours (Fig. 4A, left), followed by continuous treatment with the PARP inhibitor. No such increase in cytotoxicity was seen with 5-FU and PARP inhibitor coexposure (Fig. 4A, right).

To explore the effects of ABT-888 when cells are exposed to lower concentrations of FdUrd and 5-FU for extended periods, OVCAR-8 cells were treated with FdUrd or 5-FU plus ABT-888 for the duration of the clonogenic assay (8 days). As shown in Figure 4B, the concentrations of 5-FU and FdUrd that inhibited proliferation by 50% (IC₅₀) were reduced when cells were continuously exposed to these agents and were similar to the IC₅₀s of colon cancer cell lines that have been extensively studied with these fluoropyrimidines (Supplementary Fig. S3). Importantly, by using this exposure paradigm, ABT-888 also potentiated the effects of FdUrd but not 5-FU in OVCAR-8 (Fig. 4B) and SKOV3ip cells (Supplementary Fig. S4A). Further experiments showed that even when the clonogenic assays were done with dialyzed fetal bovine serum, which lacks thymidine and therefore enhances 5-FU’s DNA-directed cytotoxicity (39), ABT-888 still did not increase cell killing by 5-FU (Supplementary Fig. S4B), further showing that 5-FU does not exert its antiproliferative effects by causing DNA damage in these cells. In addition, as was seen with the siRNAs employed earlier, treatment with the PARP inhibitor ABT-888 did not alter formation of the FdUMP-thymidylate synthase complex in response to treatment with 5-FU and FdUrd (Supplementary Fig. S4C).

Given that cells treated continuously with 3 μmol/L ABT-888 had modestly reduced survival compared with vehicle-treated control cells, we next asked whether the cytotoxicity of ABT-888 and FdUrd was synergistic. OVCAR-8 cells were treated with increasing concentrations of FdUrd plus ABT-888 for 24 hours (Fig. 4C, left).

Figure 3. XRCC1 and PARP1 depletions sensitize to FdUrd but not 5-FU. A and B, OVCAR-8 cells were transfected with control (Luc), XRCC1 (XRCC1-2), or PARP1 (PARP1-1 and PARP1-2) siRNAs. After 48 hours, cells were trypsinized and immunoblotted for the indicated antigens (insets) or replated and exposed to the indicated concentrations of 5-FU and FdUrd for 24 hours, washed, and incubated for 7 to 10 days to allow colony formation. Data shown are a representative experiment from 2 (PARP1) or 3 (XRCC1) independent replicates. n = 3 ± SD.
washing, the initial concentrations of ABT-888 were then readded to the cultures, which were incubated until colonies formed. From these data, we conducted a formal analysis of synergy using the median effect method of Chou and Talalay (40), assuming that the agents were mutually exclusive. This analysis revealed that the combination indices for all the data points were far below 1 (Fig. 4C, right), thus indicating strong synergistic killing over a wide range of concentrations.

Of final note, these studies showed that ABT-888 concentrations as low as 0.3 μmol/L synergized with FdUrd, with higher ABT-888 concentrations even more effectively enhancing FdUrd-induced cytotoxicity. Notably, in human phase 0 clinical trials, peak plasma concentrations of ABT-888 were 0.6 and 0.9 μmol/L for patients treated with a single oral dose of 25 or 50 mg ABT-888, respectively (41), thus showing that concentrations of ABT-888 achieved in human serum following a single dose of ABT-888 synergize with FdUrd.

**ABT-888 prevents recovery from FdUrd-induced cell-cycle arrest and promotes FdUrd-induced apoptosis**

To further understand the effects of these agents on cells, we examined how ABT-888 alone, FdUrd alone, and the combination of these 2 agents (FdUrd + ABT-888) influenced the cell cycle of OVCAR-8 cells. Identical culture plates were exposed for 24 hours to ABT-888 alone, FdUrd alone, or the combination (FdUrd + ABT-888), washed, and refed with medium or with medium containing ABT-888 (for cells that were initially exposed to ABT-888). Plates were then harvested.
immediately (0 hours) or after incubation for an additional 24 or 48 hours. ABT-888 alone had no effect on the cell cycle at any time point (Fig. 5A). In contrast, 24-hour exposure to FdUrd alone caused a late S/early G1–phase arrest. Following removal of the FdUrd, the late S/early G1–phase–arrested cells moved synchronously through S phase and into G2–M. Similarly, 24-hour exposure to FdUrd + ABT-888 caused a late S/early G1–phase arrest. However, following removal of the FdUrd (and in the continued presence of ABT-888), the cells accumulated in early S phase and in G2–M. In addition, at the 48-hour time point, cells with sub-G1 levels of DNA appeared, suggesting that the cells were undergoing apoptosis. Indeed, more than 40% of the cells treated with FdUrd + ABT-888 were Annexin V-positive, another marker for apoptotic cells, at the 48-hour time point (Fig. 5B), with near-background numbers of Annexin V-positive cells in all other treated samples. Taken together, these results show that although ABT-888 does not affect cell-cycle progression in untreated cells, this PARP inhibitor dramatically slows the progression of cells with FdUrd-induced lesions and promotes apoptosis.

**ABT-888 is most effective when present during and after the FdUrd exposure**

For the experiments shown in Figure 4, ABT-888 was present during and after the FdUrd exposure period. However, it was unclear when ABT-888 exposure would most effectively synergize with FdUrd. We therefore compared a series of FdUrd and ABT-888 exposure schemes (Fig. 5C). Modestly increased cytotoxicity was observed when OVCAR-8 cells were exposed to FdUrd and ABT-888 simultaneously for 24 hours (sequence II; Fig. 5D), compared with FdUrd alone (sequence I). Similarly, exposure to FdUrd alone for 24 hours followed by continuous incubation with ABT-888 modestly increased cytotoxicity over FdUrd alone (sequence III). In contrast, the most robust killing was seen with sequences IV and V in which cells were simultaneously exposed to FdUrd...
and ABT-888, followed by continuous ABT-888 treatment after FdUrd removal.

**PARP inhibition synergizes with FdUrd in multiple ovarian cancer cell lines but not in normal cells**

To determine whether FdUrd and ABT-888 synergized in other ovarian cancer cell lines, we assessed these agents in the ovarian cancer cell lines A2780, OVCAR-3, OVCAR-5, and SKOV3ip. ABT-888 robustly potentiated the activity of FdUrd in A2780, OVCAR-3, and SKOV3ip cells, with modest effects seen in OVCAR-5 cells (Fig. 6A). Formal analyses of synergy showed that this killing was synergistic across a wide range of concentrations in the A2780, OVCAR3, and SKOV3ip cells (Supplementary Fig. S5A). In contrast, ABT-888 did not alter the cytotoxicity of FdUrd in OSEtsT/hTERT (29), which are immortalized nontransformed ovarian surface epithelial cells and WS1 human fibroblasts that undergo a limited number of replications (42).

**Comparison of FdUrd plus ABT-888 with other chemotherapy plus ABT-888 combinations**

PARP inhibition has been reported to sensitize tumor cells to multiple chemotherapy agents (28). We therefore evaluated the relative ability of ABT-888 to sensitize to various therapies that are used in the treatment of ovarian cancer. Consistent with published results, ABT-888 sensitized OVCAR-8 cells to the topoisomerase I poison topotecan (Fig. 7; ref. 28). Similarly, ABT-888 modestly increased the antiproliferative effect of the nitrogen mustard melphalan. In contrast, ABT-888 did not sensitize to the platinating agents, cisplatin, oxaliplatin, and carboplatin; the anthracycline antibiotic doxorubicin; the nucleoside analog gemcitabine; the topoisomerase II poison etoposide; or the antimitotic agent vinorelbine (Fig. 7 and Supplementary Fig. S5B). As a control, we also assessed the effects of ABT-888 on temozolomide, an alkylating agent that induces lesions repaired by BER (43). Notably, due to the profound sensitizing effect of ABT-888 to temozolomide (28), multiple clinical trials combining ABT-888 with temozolomide are now underway (44). In this head-to-head comparison, ABT-888 sensitized these cells to FdUrd as effectively as it sensitized to temozolomide (Fig. 7, compare upper left with lower right).

**Discussion**

Despite intense study for more than 5 decades, it remains unclear whether 5-FU exerts cytotoxicity primarily by disrupting DNA replication (via inhibition of thymidylate synthase...
and/or nucleotide misincorporation) or by incorporation into RNA in human tumor cell lines. In the present article, we have systematically assessed the roles of the ATR and ATM checkpoint signaling pathways and BER to understand how 5-FU kills ovarian cancer cells with the long-term goal of identifying novel therapies for this disease. Our results show that although 5-FU activates both the ATR and ATM checkpoint signaling pathways and causes DNA damage (as indicated by phosphorylation of H2AX), these pathways do not affect the survival of cells treated with 5-FU. Similarly, our studies of the BER pathway showed that disrupting this repair pathway by depleting XRCC1 or disabling PARP1 (with siRNA depletion or small-molecule inhibition) did not sensitize these cell lines to 5-FU. Taken together, these results suggest that even though 5-FU is causing DNA damage, its primary cytotoxic effect is due to the disruption of a different cellular process, which most likely depends on the incorporation of 5-FU into RNA.

Our results on the roles of the checkpoint signaling pathways in 5-FU–treated ovarian cancer cells differ from findings reported in cell lines derived from other types of cancer. In these previously published studies, disruption of ATR or Chk1 sensitized BKO, SW620, HCT116 parental, and p53−/− HCT116 cells (derived from colon cancer), Panc-1 (derived from pancreatic cancer), and HeLa cells (derived from endometrial cancer) to 5-FU (17, 21, 22, 37, 45). In contrast, we found that depletion of ATR alone, ATM alone, or even simultaneous ATM and ATR depletion did not sensitize either cell line to 5-FU. Given that in the same experiments, the ATM depletion effectively sensitized to ionizing radiation (Supplementary Fig. S1B) and that ATR depletion dramatically sensitized to FdUrd (Fig. 2A and B), it is unlikely that our results are explained by insufficient depletion of these checkpoint kinases. Instead, our findings suggest that unknown molecular differences among these cell lines may underlie these divergent findings.

In contrast to our findings with 5-FU, our studies with FdUrd found that depletion of ATR, XRCC1, and PARP1 sensitized OVCAR-8 cells to FdUrd, consistent with observations that FdUrd primarily exerts its antiproliferative effects in human cells by disrupting DNA replication (12). Nonetheless, our results differ from some of the published findings in rodent cells treated with FdUrd. Mouse embryo fibroblasts lacking DNA polymerase β (Polβ−/− cells), a polymerase that participates in the final steps of short-patch BER, and XRCC1-deficient Chinese hamster ovary cells were not more sensitive to FdUrd (12), suggesting that BER was not important for repair of the lesions induced by FdUrd. In contrast, McNiell and colleagues (46) found that expression of a catalytically inactive dominant negative APE1 mutant sensitized Chinese hamster ovary cells to FdUrd. Consistent with the latter result, we found that BER plays a critical role in the repair of FdUrd-inflicted lesions in human ovarian cancer cells.

Our finding that XRCC1 and PARP1 depletion sensitized ovarian cancer cells to FdUrd immediately suggested that small-molecule PARP inhibitors might also sensitize these cell lines to FdUrd. Indeed, both ABT-888 and AZD2281 robustly...
potentiated the antiproliferative activity of FdUrd in multiple ovarian cancer cell lines. Because ovarian cancer afflicts more than 22,000 women and kills more than 16,000 women yearly in the United States (47), new therapeutic options are needed. Given that (i) FdUrd has activity against ovarian cancer in clinical trials as a single agent, (ii) ABT-888 sensitizes to FdUrd more effectively than to other drugs often used to treat ovarian cancer, and (iii) the FdUrd plus ABT-888 combination effectively kills multiple ovarian cancer cell lines, our findings suggest that further preclinical studies that combine these agents are warranted.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

References


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