ATR Inhibition Broadly Sensitizes Ovarian Cancer Cells to Chemotherapy Independent of BRCA Status

Catherine J. Huntoon, Karen S. Flatt, Andrea E. Wahnner Hendrickson, Amelia M. Huehls, Shari L. Sutor, Scott H. Kaufmann, and Larry M. Karnitz

Abstract

Replication stress and DNA damage activate the ATR-Chk1 checkpoint signaling pathway that licenses repair and cell survival processes. In this study, we examined the respective roles of the ATR and Chk1 kinases in ovarian cancer cells using genetic and pharmacologic inhibitors in combination with cisplatin, topotecan, and veliparib. RNA interference (RNAi)–mediated depletion or inhibition of ATR sensitized ovarian cancer cells to all four agents. In contrast, while cisplatin, topotecan, and gemcitabine each activated Chk1, RNAi-mediated depletion or inhibition of this kinase in cells sensitized them only to gemcitabine. Unexpectedly, we found that neither the ATR kinase inhibitor VE-821 nor the Chk1 inhibitor MK-8776 blocked ATR-mediated Chk1 phosphorylation or autophosphorylation, while cisplatin, topotecan, and veliparib beyond the potent sensitization already caused by their deficiency in homologous recombination. Taken together, our results established that ATR and Chk1 inhibitors differentially sensitize ovarian cancer cells to commonly used chemotherapy agents and that Chk1 phosphorylation status may not offer a reliable marker for inhibition of the ATR-Chk1 pathway. A key implication of our work is the clinical rationale it provides to evaluate ATR inhibitors in combination with PARP inhibitors in BRCA1/2-deficient cells.

Introduction

Epithelial ovarian cancers are initially treated with platinum-based therapies, which induce very high response rates. Despite this initial chemoresponsiveness, more than 70% of patients will die of this disease. Accordingly, there is intense interest in identifying approaches to enhance the initial responses and/or to counter the emergence of resistance. One possible approach to increase sensitivity to chemotherapy is the pharmacological inhibition of the replication checkpoint signaling pathway. This pathway, which promotes cell survival, is activated by inhibition of DNA replication, as occurs when dNTP levels are disrupted or the replication fork encounters DNA damage. When such genotoxic stress blocks DNA replication, the continued action of helicases that unwind the DNA in front of the advancing DNA polymerases causes the accumulation of extensive regions of single-stranded DNA, which is coated with replication protein A. The replication protein A-coated single-stranded DNA attracts the kinase ATR and promotes the loading of the Rad9-Hus1- Rad1 complex onto DNA. The 9-1-1 complex and its associated protein, TopBP1, then activate ATR, which phosphorylates hundreds of substrates. Although the effects of most of these phosphorylations have not been characterized, one ATR substrate that has been intensely studied is Chk1, a kinase that phosphorylates CDC25A to block replication fork progression. Taken together, our results established that ATR and Chk1 inhibitors differentially sensitize ovarian cancer cells to commonly used chemotherapy agents and that Chk1 phosphorylation status may not offer a reliable marker for inhibition of the ATR-Chk1 pathway. A key implication of our work is the clinical rationale it provides to evaluate ATR inhibitors in combination with PARP inhibitors in BRCA1/2-deficient cells.

Authors' Affiliations: Divisions of 1Oncology Research and 2Medical Oncology, and 3Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Mayo Clinic, Rochester, Minnesota

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Corresponding Authors: Larry M. Karnitz and Scott H. Kaufmann, Division of Oncology Research, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905. Phone: 507-284-8950; Fax: 507-283-0107; E-mail: Kaufmann.Scott@mayo.edu; and Larry M. Karnitz, E-mail: Karnitz.Larry@mayo.edu doi: 10.1158/0008-5472.CAN-13-0110 ©2013 American Association for Cancer Research.
is the target of a number of inhibitors already in development (8, 9), relays the majority of the ATR signal that promotes cell survival. However, recent studies suggest that the effects of disabling ATR versus Chk1 may differ in that Chk1 inhibition might not uniformly sensitize to genotoxic drugs (11–13). These emerging results raise questions about the relative roles of ATR and Chk1 in tumor cells treated with chemotherapy agents.

Accordingly, the present studies were designed to comprehensively compare the roles of ATR and Chk1 in ovarian cancer cell lines treated with classes of agents that, despite diverse mechanisms of action, have activity in this disease. Specifically, these studies were designed to address 3 issues. First, using siRNAs and highly selective small-molecule inhibitors, we compared the effects of disabling ATR versus Chk1 in ovarian cancer cells exposed to cisplatin, gemcitabine, topotecan, and veliparib. Second, we examined the ATR/Chk1 signaling pathway looking for reliable markers of sensitization that could potentially be used in future clinical trials. Finally, given the hypersensitivity of homologous recombination (HR)-deficient ovarian cancers to cisplatin, topotecan, and PARP inhibitors (14), we investigated whether inhibition of the ATR-Chk1 pathway could further sensitize BRCA1- or BRCA2-deficient cells. Our results indicate that Chk1 inhibitors robustly sensitize to gemcitabine but not the other agents, whereas ATR inhibition sensitizes to a much broader range of chemotherapy. Importantly, interruption of ATR signaling (but not Chk1 signaling) strikingly further sensitized BRCA1- and BRCA2-deficient ovarian cancer cells to PARP inhibition, providing a potential approach for making PARP inhibitors even more effective in HR-deficient tumors.

Materials and Methods

Materials

Veliparib (ABT-888) was purchased from Enzo Life Sciences, Selleck Chemicals, or ChemieTek; VE-821 and MK-8776 were from ChemieTek; LY 2603618 was from Selleck Chemicals; and heat shock protein 90 (HSP90) from D. Scudiero, National Cancer Institute, Frederick, MD. Antibodies to various antigens were as follows: phospho-Ser139-H2AX from Millipore; CDC25A from Abcam; phospho-Ser345-Chk1, phospho-Ser296-Chk1, BRCA1, and horseradish peroxidase-linked rabbit and mouse IgGs from Cell Signaling Technology; Chk1 and Rad51 from Santa Cruz Biotechnology; phospho-Ser139-H2AX from Millipore; CDC25A from Abcam; ATR from Genetex; and heat shock protein 90 (HSP90) from D. Toft (Mayo Clinic, Rochester, MN).

Tissue culture

SKOV3 cells (V. Shridhar, Mayo Clinic) and OVCAR-8 cells (D. Scudiero, National Cancer Institute, Frederick, MD) were cultured in RPMI-1640 containing 8% FBS and 1 mmol/L glutamine. PEO1 and PEO4 cells (F. Couch, Mayo Clinic) were cultured in RPMI-1640 containing 8% FBS and 1 mmol/L glutamine (D. Scudiero, National Cancer Institute, Frederick, MD) were cultured in RPMI-1640 containing 8% FBS and 1 mmol/L glutamine. PEO1 and PEO4 cells (F. Couch, Mayo Clinic) were cultured in RPMI-1640 containing 8% FBS and 1 mmol/L glutamine.

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To assess colony formation in nontransfected OVCAR-8 and SKOV3 cells, 200 cells per well (in 6-well dishes) were plated, allowed to adhere for 4 to 6 hours, treated with the indicated agents, and allowed to form colonies for 7 to 9 days. For OVCAR-8 cells transfected with siRNAs, the indicated numbers of cells were plated. PEO1 and PEO4 cells were plated at 1000 cells per dish in 60-mm dishes, allowed to adhere overnight, treated with the indicated agents continuously, and cultured for 14 days. Following incubation, plates were stained with Coomassie Brilliant Blue and scored for colony formation (≥50 cells) manually. For clonogenic assays using nontransfected cells, percentage survivals of all individual and combination treatments were normalized to cells treated with vehicle only. For clonogenic assays using cells transfected with siRNA, percentage survivals at each drug concentration were normalized to the vehicle-treated control for the given siRNA.

Transfection

siRNAs (400 nmol/transfection) were mixed with 5 × 10^6 cells in 0.2 mL RPMI-1640 containing 8% FBS in a 0.4-cm electroporation cuvette and electroporated with two 10-mS, 280-V pulses in a BTX ECM830 square wave electroporator (Harvard Apparatus) on 2 consecutive days. The transfected cells were cultured for 48 hours before use. Rad51 SMARTpool siRNA was from Thermo Scientific. Sequences of other siRNAs (from Thermo Scientific) were: ATR-2, 5′-CCUCGUGAU-GUUGCUCUGA-3′ (15); Chk1, 5′-AAGCGUGCCGUAGACUGUC-GUCCA-3′ (16); BRCA1, 5′-GUGGUGUGGAGACUGUA-3′ (17); and luciferase, 5′-CUCAGUCAGUCUGUA-3′ (18).

Immunoblotting and cell-cycle analysis

Logarithmically proliferating cells were exposed to the indicated drugs for 4 hours, washed with PBS, and lysed in 2× SDS-PAGE sample buffer (1 × 10^7 cells/mL). Lysates (2 × 10^7 cells/lane) were separated by SDS-PAGE, transferred to Immobilon P, and blotted for the indicated antigens. For cell-cycle analyses, logarithmically proliferating OVCAR-8 cells were incubated with one or both drugs for 24 hours, released by trypsinization, and analyzed as described (19).

HR assay

OVCAR-8 cells with stable integration of pDR-GFP, an HR substrate that generates a functional GFP upon successful HR by I-SceI cleavage, were generated as described (20). For studies with siRNAs, OVCAR-8-DR-GFP cells were electroporated on day 1 with siRNA (as described above), on day 2 with siRNA plus 40 μg pCASAct plasmid (encoding I-SceI), and analyzed for GFP fluorescence on day 5.

Results

ATR depletion sensitizes to genotoxic chemotherapy more broadly than Chk1 depletion

Ovarian cancers are responsive to multiple genotoxic agents, including cisplatin, topotecan, gemcitabine, and veliparib, all of which act by disparate mechanisms.
include DNA crosslinking (cisplatin), topoisomerase I poisoning (topotecan), DNA synthesis inhibition by dNTP disruption and DNA polymerase stalling (gemcitabine), and PARP inhibition (veliparib). To address how disabling Chk1 versus ATR affects the sensitivity of ovarian cancer cells to these agents, we initially used siRNAs to deplete ATR and Chk1. As shown in Fig. 1, depletion of ATR (Fig. 1A) sensitized OVCAR-8 cells to continuous cisplatin (Fig. 1B), topotecan (Fig. 1C), and veliparib (Fig. 1D) exposure. In contrast, Chk1 depletion did not affect the cytotoxicity of these agents (Fig. 1B–D). Interestingly, neither ATR nor Chk1 depletion sensitized OVCAR-8 cells to gemcitabine under these continuous exposure conditions (Fig. 1E), possibly because gemcitabine metabolites remain trapped in the cells longer than ATR remains suppressed (about 72 hours after siRNA transfection, data not shown). In accord with this possibility, ATR and Chk1 depletion effectively sensitized the cells to a 24-hour gemcitabine exposure (Fig. 1F).

The ATR inhibitor VE-821 also sensitizes more broadly to chemotherapy

In further experiments, we explored whether ATR and Chk1 inhibitors caused effects similar to those seen with ATR and Chk1 siRNAs. For these studies we used VE-821, a potent ATR inhibitor ($K_i \sim 13 \text{ nmol/L}$) with high selectivity for ATR versus other PI3K-like kinases, including ATM (21). To inhibit Chk1, we used MK-8776 (SCH900776), which effectively inhibits Chk1 ($K_i \sim 3 \text{ nmol/L}$) and sensitizes cells to antimetabolites but does not affect the closely related kinase Chk2 (13, 22, 23). As was observed in cells depleted of ATR, VE-821 sensitized OVCAR-8 (Fig. 2A), SKOV3 (Fig. 2B), and PEO1 (Supplementary Fig. S1) ovarian cancer cells to cisplatin, topotecan, and veliparib. MK-8776, on the other hand, selectively sensitized these cell lines to gemcitabine but not the other agents (Fig. 2A and B and Supplementary Fig. S1), just as was observed with Chk1 siRNA. Consistent with these findings, parallel studies with another Chk1 inhibitor, LY2603618, showed that this agent also robustly sensitized SKOV3, OVCAR-8, and PEO1 cells to gemcitabine (Supplementary Fig. S2). Taken together, the findings in Figs. 1 and 2 indicate that (i) disruption of ATR signaling broadly sensitizes ovarian cancer cells to genotoxic chemotherapies that act by disparate mechanisms; (ii) disabling Chk1 selectively sensitizes to gemcitabine; and (iii) VE-821 and MK-8776 phenocopy the effects of depleting ATR and Chk1, respectively, suggesting that these agents are sensitizing cells by inhibiting the intended checkpoint kinases.

VE-821 and MK-8776 abrogate chemotherapy-induced cell-cycle arrest

We next tested whether these checkpoint inhibitors could override the cell-cycle arrests induced by these chemotherapy agents. Consistent with the lack of effect of PARP inhibition in cells with functional HR, veliparib minimally affected the cell cycle of OVCAR-8 cells, and co-treatment with MK-8776 or VE-821 had little additional impact (Fig. 3). In contrast, in cells exposed to cisplatin or topotecan, the addition of MK-8776 or VE-821 reduced the S-phase (cisplatin) and G2-M (cisplatin and topotecan) accumulations induced by these agents, whereas these checkpoint inhibitors modestly increased the G1 arrest induced by gemcitabine. Collectively, these results indicate that both checkpoint inhibitors effectively override the arrest induced by topotecan and cisplatin but do not allow gemcitabine-treated cells to bypass the disruption of replication caused by this antimetabolite.
VE-821 and MK-8776 do not effectively block ATR-mediated Chk1 phosphorylation and Chk1 autophosphorylation in ovarian cancer cells

The observation that VE-821 and MK-8776 abrogate the cell-cycle arrest induced by cisplatin and topotecan suggests that they are inhibiting the ATR-Chk1 signaling pathway. To further evaluate the impacts of these agents on this pathway, we next assessed their effects on ATR-mediated Chk1 phosphorylation (Ser345) and Chk1 autophosphorylation (Ser296). Consistent with previous studies of Chk1 inhibitors (9), MK-8776 (0.3 and 1 μmol/L) caused increased Chk1 Ser345 phosphorylation and H2AX Ser139 phosphorylation, a marker of DNA damage, in OVCAR-8 cells co-treated with the Chk1 inhibitor plus cisplatin, topotecan, veliparib, or gemcitabine (Fig. 4A) and in SKOV3 ovarian cells treated with gemcitabine (Fig. 4B). This increased Ser345 phosphorylation has been attributed to disruption of PP2A-mediated dephosphorylation on this site and increased DNA damage that accumulates when Chk1 cannot regulate replication (9). In contrast, the effects of MK-8776 on Chk1 autophosphorylation (Ser296) revealed unexpected results. Previous work showed that Chk1 Ser296 autophosphorylation is blocked by MK-8776 and other Chk1 inhibitors (13, 22, 23). In agreement with these earlier results, we observed that MK-8776 (0.3 and 1 μmol/L) effectively blocked gemcitabine-induced Chk1 Ser296 phosphorylation in MiaPaCa pancreatic cancer cells (Fig. 4C) and U937 leukemia cells (Fig. 4D). Surprisingly, however, MK-8776 did not prevent Chk1 Ser296 autophosphorylation in OVCAR-8 cells treated with cisplatin, and this effect was seen over a wide range of cisplatin concentrations that spanned from twice (1 μmol/L) to 10 times the IC50 (50 μmol/L; Supplementary Fig. S3). Similarly, MK-8776 did not blunt Ser296 autophosphorylation in cells exposed to gemcitabine and topotecan (Fig. 4A). Indeed, with all of the agents tested, MK-8776 actually increased genotoxin-induced Chk1 phosphorylation. MK-8776 likewise caused increased gemcitabine-induced Chk1 Ser296 phosphorylation in SKOV3 ovarian cancer cells (Fig. 4B). Taken together, the results in Fig. 4 show that MK-8776 blocks Chk1 autophosphorylation in some cells but not others.

In parallel analyses, we also evaluated the effects of the ATR inhibitor VE-821 on the ATR-Chk1 pathway in ovarian cancer cells. As reported previously (and similar to what we observed with MK-8776), VE-821 (1 and 4 μmol/L) enhanced H2AX phosphorylation on Ser139 induced by topotecan and cisplatin in OVCAR-8 cells (Fig. 4A), suggesting that ATR inhibition caused the accumulation of additional DNA damage. Surprisingly, VE-821 did not block ATR-mediated Chk1 Ser296 autophosphorylation triggered by gemcitabine, topotecan, or cisplatin (Fig. 4A). Comparable results were also seen in gemcitabine-treated SKOV3 cells, even at concentrations up to 6 μmol/L VE-821 (Fig. 4B). Analyses of the effects of VE-821 in other cell lines revealed additional complexity. Whereas VE-821 (1 and 4 μmol/L) did not diminish Chk1 Ser296 (or Ser296) phosphorylation in MiaPaCa cells (Fig. 4C), the higher VE-821 concentration did disrupt these phosphorylation events in U937 cells (Fig. 4D). These results show that VE-821 does not effectively disrupt ATR-mediated Chk1 phosphorylation in several cell types, including ovarian cancer cells.
VE-821 and MK-8776 disrupt chemotherapy-induced CDC25A degradation

To further examine the impact of ATR and Chk1 inhibitors on this signaling pathway, we assessed the effects of MK-8776 and VE-821 on levels of CDC25A, a Chk1 substrate that is targeted for proteasomal degradation following Chk1-mediated phosphorylation. As expected for agents that activate Chk1, gemcitabine, topotecan, and cisplatin caused decreases in CDC25A levels (Fig. 4A). These genotoxin-induced reductions of CDC25A were blocked by MK-8776 and VE-821, thus showing that even though these checkpoint inhibitors did not block (and in some cases stimulated) Chk1 phosphorylation, they still disrupted the checkpoint signal.

Disabling ATR disrupts HR repair, a pathway that protects cells from cisplatin, topotecan, and veliparib, and further sensitizes cells with disabled HR to these agents

Our finding that disabling Chk1 did not sensititize to cisplatin, topotecan, or veliparib indicates that other ATR substrates help protect cells from the lesions induced by these agents. Because ATR also phosphorylates and regulates proteins that participate in HR repair, such as BRCA1 (reviewed in ref. 24), and because cisplatin, topotecan, and veliparib cause damage that is repaired by HR (25–28), we reasoned that ATR might participate in HR. Consistent with this idea, ATR depletion reduced HR-mediated repair of DR-GFP, a stably integrated HR substrate (Fig. 5A), following transfection of the I-SceI nuclease that cleaves between nonfunctional GFP repeats, thus promoting HR repair.

To examine potential interactions between ATR and HR, we next asked how disabling HR by depleting BRCA1 (Fig. 5B), alone and in combination with ATR or Chk1 inhibition, affected responses to these agents. These studies revealed several noteworthy findings. First, BRCA1 depletion did not sensitize to gemcitabine (Fig. 5C), consistent with a previous report (26), but did robustly sensitize to cisplatin, topotecan, and veliparib (Fig. 5D–G). Interestingly, these results show that ATR depletion—but not Chk1 depletion—sensitizes to the same agents that cause damage repaired by HR (i.e., cisplatin, topotecan, and veliparib—see Fig. 1). These results, therefore, suggest that ATR regulation of HR contributes to cell survival more than ATR-mediated activation of Chk1 in cells treated with agents that induce lesions repaired by HR. Second, even when BRCA1 was depleted, MK-8776 did not further sensitize cells to any of the agents (Fig. 5C–F), indicating that even when HR was disabled, Chk1 did not facilitate survival. Third, MK-8776 could still robustly sensitize BRCA1-depleted cells to gemcitabine, although this sensitization was no greater than in control (Luc) cells (Fig. 5C). Fourth, even when HR was disabled by BRCA1 depletion, VE-821 additionally sensitized cells to cisplatin and topotecan (Fig. 5D and E). Fifth, VE-821 was particularly effective at further sensitizing BRCA1-depleted cells to veliparib (Fig. 5F), a result that was also observed in BRCA1-depleted SKOV3 cells (Fig. 5G and Supplementary Fig. S4). Taken together, these results indicate that even in cells with defects in HR, ATR still plays a critical role in promoting the survival and proliferation of cells exposed to cisplatin,
topotecan, and especially veliparib, suggesting that in addition to regulating HR, ATR has additional roles in protecting tumor cells from damage inflicted by these agents.

**Discussion**

These studies were designed to compare the impact of disabling ATR versus Chk1 using siRNA or small-molecule inhibitors in ovarian cancer cells exposed to chemotherapy agents that are representatives of 4 classes of agents with activity in this disease. This analysis showed that the ATR inhibitor VE-821, like ATR siRNA, sensitized to a wide range of genotoxic stresses. In contrast, Chk1 depletion, like Chk1 inhibition, showed a much more restricted sensitization pattern. These observations have important implications for current efforts to develop Chk1 and ATR inhibitors as described in greater detail below.

Initial studies of ATR and Chk1 inhibitors used agents such as caffeine or UCN-01, which inhibit ATR or Chk1, respectively (29–32), but have subsequently been shown to inhibit multiple enzymes (33–37). More recent studies have focused on increasingly selective kinase inhibitors. For example, the Chk1 inhibitor AZD7762 sensitizes to a wide range of anticancer therapies, including gemcitabine, topotecan, cisplatin, ionizing radiation, and even the microtubule disruptor paclitaxel (38–42). Notably, however, in addition to potently inhibiting Chk1 ($K_i \sim 4$ nmol/L), AZD7762 also inhibits Chk2 with similarly potency and shows less than 10-fold selectivity for multiple members of the CAMK, AGC, and Src families of kinases (38). Thus, some of the effects of this agent may be attributable to inhibition of other kinases. Similarly, VE-821, one of the first selective ATR inhibitors to be reported, also sensitizes cells to multiple agents including cisplatin, camptothecin, etoposide, and ionizing radiation (21). Therefore, even though these Chk1 and ATR inhibitors sensitize to similar types of genotoxic chemotherapy agents, it remains unclear whether these overlapping sensitization profiles are due solely to Chk1 and ATR inhibition or whether they are caused by inhibition of other kinases. The present studies provide insight into this question by first comparing the effects of ATR and Chk1 depletion (using siRNAs) and then conducting a head-to-head comparison of VE-821 with MK-8776, an agent identified based on its ability to selectively inhibit Chk1 relative to Chk2 (22).

When the effects of ATR versus Chk1 siRNAs were compared, ATR knockdown sensitized cells to cisplatin, topotecan, gemcitabine, and veliparib (Fig. 1). Consistent with the ATR siRNA results, VE-821 also sensitized multiple ovarian cancer cell lines to these same agents (Fig. 2). In marked contrast, Chk1 depletion only sensitized to gemcitabine (Fig. 1). Similarly, even though MK-8776 effectively overrode the cell-cycle arrests induced by topotecan and cisplatin (thus showing effective Chk1 inhibition—Fig. 3), this Chk1 inhibitor only sensitized to gemcitabine (Fig. 2). Taken together, these results indicate that ATR protects ovarian cancer cells from multiple genotoxic stresses, whereas the role of Chk1 appears limited to gemcitabine, a result consistent with recent reports suggesting that MK-8776 preferentially sensitizes to the antimetabolites hydroxyurea, gemcitabine, and cytarabine (13, 22).

One question that emerges from these studies is why ATR and Chk1 have such different prosurvival effects in cells exposed to genotoxins that act by disparate mechanisms. With the exception of veliparib, all of these agents disrupt DNA replication and activate checkpoints that block cell-cycle progression, events that require Chk1 signaling. Nonetheless,
disabling Chk1 only sensitized to gemcitabine, suggesting that other ATR-regulated events are important for the other agents. Indeed, our studies raise the possibility that one such event may be the mobilization of the HR machinery because the agents that cause damage repaired by HR (cisplatin, topotecan, and veliparib) all require ATR—but not Chk1—to promote survival. Notably, however, because ATR inhibition further sensitizes cells with defective HR to cisplatin, topotecan, and veliparib (see Fig. 5F and G) or BRCA1 depletion (see Fig. 5F and G) or BRCA1 siRNA, Forty-eight hours after transfection, cells were trypsinized and used to analyze BRCA1 expression (B; OVCAR-8 cells) and for clonogenic assays (C–G). For clonogenic assays, cells were plated, allowed to adhere for 6 hours, and treated with 0.3 μmol/L MK-8776 or 1 μmol/L VE-821 plus gemcitabine (C) cisplatin (D), topotecan (E), or veliparib (F, G) for 8 days. A representative experiment from 3 independent experiments is shown.

Several studies have addressed how disabling Chk1 sensitizes cells to replication stress, but no unifying picture has emerged. On the one hand, inappropriate progression through S-phase, premature exit from G2, and mitotic catastrophe have been proposed as the mechanism by which cells die when Chk1 is inhibited during replication stress, especially when p53 signaling is disabled (reviewed in ref. 9). In contrast, other studies suggest that override of these checkpoints does not correlate with toxicity (43), and consistent with these prior findings, we observed that disabling Chk1 actually augmented gemcitabine–induced arrest in G1–S (Fig. 3) while at the same time sensitizing to gemcitabine. On the other hand, recent studies found that stalled replication forks were cleaved by the endonucleases MRE11 (44) or MUS81 (45) when Chk1 was disabled. This aberrant cleavage then caused replication fork collapse, the accumulation of double-stranded DNA breaks, and cell death. Given these disparate findings, it remains unclear whether these and/or other mechanisms participate in the toxicity of the gemcitabine + MK-8776 combination in ovarian cancers, but future studies that address these questions may help identify potential biomarkers for a clinical trial of such a drug combination.

Our studies to further characterize the effects of these checkpoint inhibitors on ovarian cancer cells revealed several unexpected findings. Previous studies showed that MK-8776 and other Chk1 inhibitors block Chk1 autophosphorylation on Ser296 (38, 46–48) and that VE-821 abrogates ATR-mediated Chk1 Ser345 phosphorylation (21), suggesting that these phosphorylation events may provide an effective way to assess
disruption of this signaling pathway in clinical trials (9, 48). The present studies, however, show that even when checkpoint inhibitors override the checkpoint signal (as shown by CDC25A preservation and cell-cycle arrest—Figs. 3 and 4), these Chk1 phosphorylation events may not be reliable markers of pathway inhibition. In particular, VE-821 concentrations that sensitized to cisplatin, topotecan, or gemcitabine did not block ATR-mediated Chk1 Ser296 phosphorylation in ovarian cancer cells (Fig. 4A and B) even though VE-821 blocked this phosphorylation in U937 leukemia cells (Fig. 4D). In a similar vein, MK-8776 concentrations that enhanced gemcitabine-induced cytotoxicity in ovarian cancer cells failed to inhibit Chk1 autophosphorylation on Ser296 (Fig. 4A and B) even though the expected effects of MK-8776 on Chk1 Ser296 phosphorylation were readily detected in pancreatic cancer and leukemia cell lines (Fig. 4C and D). Collectively, our observations raise the possibility that these Chk1 sites might not be appropriate biomarkers to assess pathway inhibition in all cell types. Equally important, the ability of VE-821 to sensitize cells to cisplatin and topotecan at concentrations that do not inhibit Chk1 Ser296 phosphorylation suggests that ATR inhibition might sensitize cells by altering phosphorylation of other, currently unappreciated substrates. Whether phosphorylation of these substrates is more sensitive than phosphorylation of Chk1, a situation analogous to differential effects of rapamycin on phosphorylation of substrates by the ATR-related kinase mTOR (49, 50), remains to be explored.

Emerging data suggest that high-grade serous ovarian cancer, the most common histologic subtype, can be categorized into tumors with defects in HR (which includes mutations in BRCA1 and BRCA2) and tumors that are proficient in HR (14). Importantly, our results show that although MK-8776 does not further sensitize cells with HR defects to any of the genotoxic chemotherapies tested here, this agent still sensitizes deficient in BRCA1 (OVCAR-8 treated with siRNA, Fig. 5C) or BRCA2 (PEO1, Supplementary Fig. S1) to gemcitabine. In stark contrast, even in cells with defective HR, which are hypersensitive to cisplatin, topotecan, and veliparib, VE-821 further sensitized the cells to these chemotherapy agents (Fig. 5).

Because Chk1 was the first ATR substrate identified and was shown to mediate some of the effects of ATR activation, much of the effort in drug development has focused on Chk1 inhibitors. The present demonstration that VE-821, like ATR siRNA, sensitizes to a much broader range of genotoxic stresses, including highly active anticancer agents such as cisplatin, topoisomerase I poisons, and veliparib, suggests that further investigation of ATR inhibitors and their mechanism of sensitization might also be worthwhile, especially in cancers with defects in HR.

Disclosure of Potential Conflicts of Interest

S.H. Kaufmann received a commercial research grant from Schering-Plough for correlative studies of a clinical trial of MK-8776 ($10,000 or more). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Huntoon, K. Flatten, A. Wahner-Hendrickson, A. Huelbs, S. Kaufmann, L. Karnitz
Development of methodology: C. Huntoon, S. Kaufmann, L. Karnitz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Huntoon, K. Flatten, A. Wahner-Hendrickson, A. Huelbs, S. Sutton, S. Kaufmann, L. Karnitz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kaufmann, L. Karnitz
Writing, review, and/or revision of the manuscript: S. Kaufmann, L. Karnitz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Huntoon, K. Flatten, S. Kaufmann, L. Karnitz
Study supervision: S. Kaufmann, L. Karnitz

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