PARP1 Trapping and DNA Replication Stress Enhance Radiosensitization with Combined WEE1 and PARP Inhibitors

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Abstract

KRAS mutations in non–small cell lung cancer (NSCLC) cause increased levels of DNA damage and replication stress, suggesting that inhibition of the DNA damage response (DDR) is a promising strategy for radiosensitization of NSCLC. This study investigates the ability of a WEE1 inhibitor (AZD1775) and a PARP inhibitor (olaparib) to radiosensitize KRAS-mutant NSCLC cells and tumors. In addition to inhibiting the DDR, these small-molecule inhibitors of WEE1 and PARP induce DNA replication stress via nucleotide exhaustion and PARP trapping, respectively. As monotherapy, AZD1775 or olaparib alone modestly radiosensitized a panel of KRAS-mutant NSCLC lines. The combination of agents, however, significantly increased radiosensitization. Furthermore, AZD1775-mediated radiosensitization was rescued by nucleotide repletion, suggesting a mechanism involving AZD1775-mediated replication stress. In contrast, radiosensitization by the combination of AZD1775 and olaparib was not rescued by nucleosides. Whereas both veliparib, a PARP inhibitor that does not efficiently trap PARP1 to chromatin, and PARP1 depletion radiosensitized NSCLC cells as effectively as olaparib, which does efficiently trap PARP, only olaparib potentiated AZD1775-mediated radiosensitization. Taken together, these mechanistic data demonstrate that although nucleotide depletion is sufficient for radiosensitization by WEE1 inhibition alone, and inhibition of PARP catalytic activity is sufficient for radiosensitization by olaparib alone, PARP1 trapping is required for enhanced radiosensitization by the combination of WEE1 and PARP inhibitors.

Implications: This study highlights DNA replication stress caused by nucleotide depletion and PARP1 trapping as an important mechanism of radiosensitization in KRAS-mutant tumors and supports further development of DNA replication as a therapeutic target.

Introduction

Although EGFR inhibitors such as erlotinib and osimertinib as well as the ALK inhibitor crizotinib have been approved for the treatment of non–small cell lung cancers (NSCLC) harboring EGFR mutations and ALK or ROS1 rearrangements, respectively, there is no targeted therapy for KRAS-mutant NSCLC, which accounts for 30% of patients. As mutations in KRAS lead to both DNA damage response pathways and therefore may be sensitive to therapies targeting DNA repair in combination with radiation therapy (1–4). Specifically, WEE1 phosphorylates and inhibits CDK1 and CDK2 to maintain the intra-S and G2 cell-cycle checkpoints in response to DNA damage. WEE1 also promotes homologous recombination (HR) through a mechanism that is likely dependent on suppression of CDK1 activity (5). Furthermore, through suppression of CDK1/2 activity, WEE1 regulates DNA replication by preventing aberrant origin firing and subsequent nucleotide shortage and replication stalling (6). Likewise, PARP1 has several functions in the DNA damage response, including promotion of base excision repair (BER; refs. 7, 8), and alternative end joining (9, 10), as well as stabilization and restart of stalled DNA replication forks (11, 12).

Small-molecule inhibitors of WEE1 and PARP are in various phases of clinical development. AZD1775, an inhibitor of the WEE1 kinase, is a first-in-class agent that is the subject of several clinical trials investigating its activity as monotherapy as well as in combination with chemotherapy and radiation. Several PARP inhibitors are in advanced stages of clinical development (e.g., talazoparib and veliparib) and several have received FDA approval (i.e., olaparib, rucaparib, and niraparib). Despite the canonical function of WEE1 in the G2-phase cell-cycle checkpoint, inhibition of HR by WEE1 inhibition is the most likely mechanism of sensitization to chemoradiation (13). Furthermore, DNA replication stress induced by WEE1 inhibition as a result of nucleotide depletion (6, 14) is a key contributor to both the monotherapy and radiosensitizing activity (15) of WEE1 inhibition. Although...
PARP1 plays a role in BER and DNA replication fork stabilization and restart, the cytotoxic activity of PARP inhibitors is due to trapping of PARP on chromatin (16–18). PARP is rapidly recruited to DNA damage sites, and its dissociation requires auto–ADP-ribosylation and thus PARP catalytic activity. By inhibiting PARP activity, small-molecule inhibitors of PARP also prevent the dissociation of PARP from DNA damage sites on chromatin. Trapped PARP interferes with DNA replication, resulting in fork stalling, which, if left unresolved, may ultimately lead to replication fork collapse. Although PARP1 trapping has been implicated in both the monotherapy and chemosensitizing activities of PARP inhibitors (especially temozolomide; ref. 19), its contribution to radiosensitization was previously unknown.

Given the mechanisms of action of both WEE1 and PARP inhibitors on different aspects of DNA replication (e.g., nucleotide depletion and PARP1 trapping, respectively), we investigated the potential contribution of DNA replication stress to the interaction between WEE1 and PARP inhibition on radiosensitization. We began by determining radiosensitization by the combination of AZD1775 and olaparib in KRAS-mutant NSCLC cells. We found that radiation sensitivity was enhanced by this combination both in vitro and in vivo, and in association with DNA replication stress. We went on to investigate the mechanisms of DNA replication stress by assessing the contribution of nucleotide shortage to sensitization via exogenous nucleoside repletion. Furthermore, we assessed the contribution of PARP1 trapping versus PARP catalytic inhibition to sensitization by comparing a PARP inhibitor that causes PARP1 trapping (olaparib) with those PARP inhibitors that do not efficiently cause PARP1 trapping, but do inhibit PARP catalytic activity (veliparib and PARP1 siRNA).

Materials and Methods

Cell culture, transfections, and drug solutions

Calu-6 and H23 cells were obtained from and authenticated (via short tandem repeat profiling) by the ATCC (2015). Cells were cryopreserved within 3 months of authentication. H1703 KRAS isoegenic cells were a gift from Dr. Henning Willers, Harvard Medical School, Boston, MA (20). Cells were grown in either MEM (Calu-6) or RPMI 1640 media (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals). Specific knockdown of PARP1 was performed with Oligofectamine Transfection Reagent (Roche) as per the manufacturer’s protocol using a pool of PARP1 siRNA’s purchased from Dharmacon. EmbryoMAX nucleoside solution (Millipore) was used at a 1:12.5 dilution concurrently with AZD1775 and olaparib. For in vitro experiments, AZD1775, olaparib (AstraZeneca), and veliparib (NCI, Cancer Therapy Evaluation Program) were each dissolved in DMSO (Sigma) and stored in aliquots at –20°C. For in vivo experiments, AZD1775 was suspended in 0.5% methylcellulose (Sigma) and stored for a maximum of 5 days at room temperature with constant stirring. Olaparib was diluted as needed in 10% 2-hydroxypropyl-ß-cyclo-dextrin (Sigma).

Clonogenic survival assays

Cells treated with drugs and/or radiation were processed for clonogenic survival as described previously (21, 22). Unless otherwise indicated, AZD1775 and olaparib were given for 25 hours, beginning 1 hour prior to radiation. Radiation survival curves were normalized for drug toxicity, and the radiation enhancement ratio was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after drug exposure (23). A value significantly greater than 1 indicates radiosensitization. Cytotoxicity in the absence of radiation treatment was calculated by normalizing the plating efficiencies of drug-treated cells to non–drug-treated cells.

Detection of pSer10 histone H3 or γH2AX by flow cytometry

Treated cells were trypsinized, washed with ice-cold PBS, and fixed at a concentration of 2 × 10⁶ cells/mL in ice-cold 70% ethanol. For pSer10 histone H3 (γH3) analysis, samples were first incubated with a rabbit anti-phH3 antibody (#06-570, EMD Millipore) diluted 1:133 in PBS buffer containing 5% FBS and 0.5% Tween-20 (Sigma) overnight at 4°C, followed by incubation with a FITC-conjugated secondary antibody (Sigma Biochemical) as described previously (24). Normal and premature mitoses were defined as the fraction of pH3-positive cells with either a 4N (normal) or sub-4N (premature) DNA content. For γH2AX analysis, samples were incubated with a mouse monoclonal anti-γH2AX antibody (JBW301, EMD Millipore) diluted 1:500 in PBS buffer containing 1% FBS and 0.2% Triton X-100 (Sigma), followed by incubation with an FITC-conjugated anti-mouse secondary antibody as described previously (25). Samples were then stained with propidium iodide to assess total DNA content and analyzed on a FACSscan flow cytometer (Becton Dickinson) with FlowJo software (Tree Star). For quantification of γH2AX positivity, a gate was arbitrarily set on the control, untreated sample to define a region of positive staining for γH2AX of approximately 5% to 10%. This gate was then overlaid on the treated samples.

Immunoblotting and fractionation of PARP1–chromatin complexes

Whole-cell lysates were prepared in cold SDS lysis buffer (10 mmol/L Tris, 2% SDS) supplemented with both PhosSTOP phosphatase inhibitor and Complete protease inhibitor cocktails (Roche) as described previously (22). To assess PARP1 bound to chromatin, nuclei were isolated from 106 cells/mL in ice-cold hypotonic buffer (50 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.34 mol/L sucrose, 10% glycerol, 1 mmol/L DTT, 0.1% Triton X-100) supplemented with protease inhibitors followed by slow-speed centrifugation (1,300 g × g at 4°C for 4 minutes). Washed nuclei were then lysed in ice-cold buffer B (3 mmol/L EDTA, 0.2 mol/L EGTA, 1 mmol/L DTT) supplemented with protease inhibitors, and insoluble chromatin was collected by centrifugation (4 minutes, 1,700 × g at 4°C), washed once in buffer B, centrifuged again under the same conditions, and processed for Western blot analysis as described previously (26). Densitometric analyses of immunoblots were carried out using ImageJ software (NIH, Bethesda, MD).

Antibodies

The following antibodies were used: rabbit monoclonal anti–pY15-Cdk2 (10A11; #4539), rabbit polyclonal anti-PARP1 (#9542), mouse monoclonal anti-Histone H3 (96C10; #3638) and rabbit monoclonal anti-GAPDH (14C10; #2118) from Cell Signaling Technology; rabbit polyclonal anti–pS10-Histone H3 (06-570), mouse monoclonal anti-γH2AX (JBW301; 05-636) and rabbit polyclonal anti-CDC2/CDK1 (06-966) from Millipore; mouse monoclonal anti-PAR (10H, Ab1) from either Calbiochem or Abcam; mouse monoclonal anti-RPA32 (918; ab2175) from Abcam; and rabbit polyclonal anti-phospho-RPA32 (S4/S8;
A300-245A) and anti–phospho-RPA32 (S33; A300-246A-M) from Bethyl Laboratories.

**Immunofluorescence**

For immunofluorescence experiments, Calu-6 cells were grown on coverslips and treated in 12-well dishes. Following treatment, cells were fixed with 3.7% paraformaldehyde and stained with a mouse monoclonal γH2AX antibody (JBW301, EMD Millipore) and DAPI (4',6-diamidino-2-phenylindole) as described previously (27). Samples were imaged with an Olympus IX71 FluoView confocal microscope (Olympus America) with a 60× oil objective. Fields were chosen at random based on DAPI staining. For quantitation of γH2AX foci and pan–γH2AX-stained cells, at least 100 cells from each of four independent experiments were visually scored for each condition. Cells with 10 or more γH2AX foci were scored as positive.

**Irradiation**

Irradiations were performed using a Philips RT250 (Kimtron Medical) at a dose rate of approximately 2 Gy/minute at the University of Michigan Comprehensive Cancer Center Experimental Irradiation Shared Resource. Dosimetry was performed using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration. For tumor irradiation, animals were anesthetized with isoflurane and positioned such that the apex of each flank tumor was at the center of a 2.4-cm aperture in the secondary collimator, with the rest of the mouse shielded from radiation.

**Tumor growth studies**

Animals were handled in accordance with protocols approved by the University of Michigan Committee for Use and Care of Animals. Calu-6 cells (5 × 10⁶) were suspended in a 1:1 mixture of 10% FBS-MEM/Matrigel (BD Biosciences) and injected subcutaneously, bilaterally into the flanks of 3- to 5-week-old, female athymic nude mice (Harlan). Treatment was initiated when the average tumor volume reached 100 mm³ and consisted of olaparib (50 mg/kg, once daily; 2 hours preradiation; Monday–Friday) AZD1775 (120 mg/kg; once daily; 1 hour preradiation; Monday–Friday), and radiation (2 Gy/fraction; Monday–Friday).

Figure 1. Radiosensitization by combined WEE1 and PARP inhibition. Calu-6, H23, and H1703 KRAS-mt and -wt NSCLC cell lines were treated with AZD1775 (Calu-6 cells: 150 nmol/L, other cell lines: 100 nmol/L) and/or olaparib (H23 cells: 300 nmol/L, other cell lines: 1 μmol/L) beginning 1 hour prior to radiation (RT; 0–10 Gy). Twenty-four hours postradiation, cells were processed for clonogenic survival. Data from a single representative experiment for each cell line are shown (A–D). The complete dataset and statistical analyses are provided in Table 1A.
Table 1. Radiosensitization and cytotoxicity by combined WEE1 and PARP inhibition

<table>
<thead>
<tr>
<th>A. Condition</th>
<th>RER</th>
<th>Cytotoxicity</th>
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<tbody>
<tr>
<td>Calu-6</td>
<td></td>
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<tr>
<td>AZD1775 (150 nmol/L)</td>
<td>1.45 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74 ± 0.12</td>
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<tr>
<td>Olaparib (1 μmol/L)</td>
<td>1.22 ± 0.07</td>
<td>1.14 ± 0.21</td>
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<td>AZD1775 + olaparib</td>
<td>1.88 ± 0.08&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.61 ± 0.06</td>
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<tr>
<td>H23</td>
<td></td>
<td></td>
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<tr>
<td>AZD1775 (100 nmol/L)</td>
<td>1.09 ± 0.04</td>
<td>0.87 ± 0.09</td>
</tr>
<tr>
<td>Olaparib (300 nmol/L)</td>
<td>1.29 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03 ± 0.15</td>
</tr>
<tr>
<td>AZD1775 + Veliparib</td>
<td>1.49 ± 0.06&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td>H1703 KRAS mt</td>
<td></td>
<td></td>
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<tr>
<td>AZD1775 (100 nmol/L)</td>
<td>1.23 ± 0.04</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>Olaparib (1 μmol/L)</td>
<td>1.15 ± 0.10</td>
<td>0.94 ± 0.11</td>
</tr>
<tr>
<td>AZD1775 + olaparib</td>
<td>1.65 ± 0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.75 ± 0.08</td>
</tr>
<tr>
<td>H1703 KRAS wt</td>
<td></td>
<td></td>
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<tr>
<td>AZD1775 (100 nmol/L)</td>
<td>1.31 ± 0.10</td>
<td>0.81 ± 0.09</td>
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<tr>
<td>Olaparib (1 μmol/L)</td>
<td>1.14 ± 0.06</td>
<td>0.92 ± 0.05</td>
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<tr>
<td>AZD1775 + olaparib</td>
<td>1.46 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.73 ± 0.08</td>
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<tr>
<td>B. Calu-6</td>
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<tr>
<td>AZD1775 (150 nmol/L)</td>
<td>1.40 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.75 ± 0.05</td>
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<tr>
<td>+ nucleosides</td>
<td>1.05 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.83 ± 0.04</td>
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<tr>
<td>Olaparib (1 μmol/L)</td>
<td>1.22 ± 0.06</td>
<td>0.98 ± 0.07</td>
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<tr>
<td>+ nucleosides</td>
<td>1.24 ± 0.08</td>
<td>1.10 ± 0.06</td>
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<tr>
<td>AZD1775 + olaparib</td>
<td>1.67 ± 0.16</td>
<td>0.77 ± 0.04</td>
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<tr>
<td>+ nucleosides</td>
<td>1.60 ± 0.11</td>
<td>0.88 ± 0.10</td>
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<tr>
<td>C. Calu-6</td>
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<td></td>
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<tr>
<td>N.S. siRNA</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+ AZD1775</td>
<td>1.48 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td>+ PARP1 siRNA</td>
<td>1.10 ± 0.02</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>+ AZD1775</td>
<td>1.55 ± 0.13&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.69 ± 0.15</td>
</tr>
<tr>
<td>+ Veliparib</td>
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<tr>
<td>+ Veliparib</td>
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</tr>
<tr>
<td>+ AZD1775</td>
<td>1.47 ± 0.06</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>+ Veliparib</td>
<td>1.20 ± 0.14</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>+ AZD1775</td>
<td>1.47 ± 0.10</td>
<td>0.64 ± 0.05</td>
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NOTE: A, Calu-6, H23, and H1703 KRAS-mt and -wt NSCLC cell lines were treated with AZD1775 and/or olaparib beginning 1 hour prior to radiation (0–10 Gy). Twenty-four hours postradiation, cells were processed for clonogenic survival. Data are either the mean radiation enhancement ratio (RER) ± SEM of the mean clonogenic survival ± SEM for n = 4–6 independent experiments. Cytotoxicity in the absence of radiation treatment was calculated by normalizing the plating efficiencies of drug-treated to non-drug-treated cells. B. Survival data from Calu-6 cells treated with AZD1775 and/or olaparib in the presence of exogenous nucleosides were calculated as described above (n = 4). C. Survival data from PARP1-depleted Calu-6 cells treated with AZD1775 or AZD1775 + cells treated with AZD1775 and/or Veliparib were calculated as described above (n = 3–6).<sup>P < 0.05 (one-way ANOVA) vs. control<sup>8</sup>, AZD1775<sup>c</sup>, olaparib<sup>b</sup>, N.S. control<sup>9</sup>, or PARP1 siRNA</sup>.

for 1 cycle. AZD1775 and olaparib were administered via oral gavage. Tumor size was measured two times per week. Tumor volume (TV) was calculated according to the equation: TV = π/6 (ab<sup>2</sup>)<sup>3</sup>, where a and b are the longer and shorter dimensions of the tumor, respectively. Measurements were made until day 35 or until the tumor volume increased by approximately a factor of 4, whichever occurred first. Time to tumor volume doubling was calculated as the earliest day on which the tumor volume is at least twice as large as on the first day of treatment. Log-rank tests were conducted to compare tumor volume doubling between treatment arms.

**Results**

Before assessing the abilities of combined WEE1 and PARP inhibition to radiosensitize KRAS-mutant NSCLC cells, we first determined the cytotoxicity of AZD1775 and olaparib in the absence of radiation. Although olaparib alone (1 μmol/L) was nontoxic, AZD1775, either alone or in combination with olaparib, produced a concentration-dependent decrease in survival with 100 to 150 nmol/L AZD1775 causing moderate toxicity (25%–40% cell killing) when given in combination with olaparib (Supplementary Fig. S1A–S1D). Using moderately active concentrations of AZD1775 and olaparib, administered 1 hour prior to and 24 hours postradiation, we next assessed radiosensitization in a panel of NSCLC cell lines. Although treatment with either agent alone produced modest radiosensitization, the combination of AZD1775 and olaparib produced significantly greater radiosensitization than either agent alone in each of the KRAS-mutant cell lines (Calu-6, H23, and H1703 cells constructed to express mutant KRAS), as well as in parental KRAS wild-type H1703 cells (Fig. 1A–D, Table 1A). Taken together, these results demonstrate that the combination of WEE1 and PARP inhibitors with radiation is an active therapeutic strategy in KRAS-mutant lung cancer cells.

We next evaluated the ability of AZD1775 and olaparib to inhibit their respective downstream targets, namely phosphorylated CDK1 (γH2AX) and PAR (poly (ADP-ribose)), in Calu-6 and KRAS-mutant H1703 cells. As anticipated, olaparib alone or in combination with AZD1775/radiation reduced PAR levels, a result consistent with inhibition of PARP catalytic activity (Fig. 2A and B). AZD1775 caused an increase in PAR, suggesting greater DNA damage and/or DNA replication stress. Consistent with inhibition of WEE1 kinase activity, AZD1775 reduced pCDK1 (Y15) levels. The combination of AZD1775 and olaparib did not further potentiate inhibition of either respective target, suggesting that more effective target engagement is not the mechanism of combination radiosensitization. As WEE1 inhibition can lead to aberrant origin firing, nucleotide depletion, and subsequent replication stress (6), we also assessed pRPA (S33) and pRPA (S4/8), activated forms of RPA associated with extended regions of single-stranded DNA and replication-associated DNA damage (28). AZD1775 alone or in combination with olaparib caused a dramatic increase in both pRPA (S33) and pRPA (S4/8), suggesting the presence of DNA replication stress.

Given the canonical role of WEE1 in the G2 checkpoint, we also assessed the effects of AZD1775 and olaparib on radiation-induced phosphorylated Histone H3 (pHistoneH3), a marker of early mitosis. We found that AZD1775 alone or in combination with olaparib abrogated the radiation-induced G2 checkpoint as evidenced by an increase in pHistoneH3 positivity in Calu-6, H23 and both H1703 KRAS-mutant and wild-type cells (Fig. 2C and D, Supplementary Fig. S2A–S2C). In addition, AZD1775 caused premature mitotic entry (pHistoneH3-positive cells with incompletely replicated DNA) in Calu-6 and both H1703 KRAS-mutant and wild-type cells, a result consistent with impaired DNA replication as well as G2 checkpoint abrogation. Results from prior studies, however, suggest that although G2 checkpoint abrogation illustrates the biological activity of AZD1775 under the conditions used in this study, it is likely not a critical mechanism of radiosensitization by WEE1 inhibition (13, 29).

As both WEE1 and PARP function in DNA repair (5, 30) as well as DNA replication (6, 14), we next assessed the effects of AZD1775 and olaparib on the radiation-induced DNA damage response by measuring γH2AX staining, a surrogate marker for both unrepaired DSBs and replication stress (31–33). In the absence of radiation, AZD1775 alone or in combination with olaparib caused an increase in total γH2AX levels (Fig. 3A–C). In
response to radiation alone, total γH2AX levels peaked within 2 hours and then resolved over time, a response consistent with the induction and repair of radiation-induced DSBs (Fig. 3A and B; Supplementary Fig. S3A and S3B). Treatment with olaparib delayed the initial resolution of total γH2AX (2–6 hours) in most models, but γH2AX levels 24 hours postradiation in combination with olaparib were similar to radiation alone. In contrast, AZD1775 alone or in combination with olaparib persisted impaired the resolution of radiation-induced γH2AX, resulting in significantly higher levels 24 hours postradiation in Calu-6 cells with similar, although less pronounced effects in H23 and H1703 KRAS wild-type cells (Supplementary Fig. S3A and S3B). In the H1703 KRAS-mutant cells, however, the increase in total γH2AX levels in response to AZD1775 (with or without olaparib) and radiation was attributable to the accumulation of AZD1775-mediated DNA replication stress (31, 32). Over time, AZD1775 alone or in combination with olaparib caused an increase in high intensity γH2AX staining (16–24 hours; Fig. 3C and D). In response to radiation, AZD1775 alone or in combination with olaparib caused a significant increase in high intensity γH2AX staining in Calu-6, H1703 KRAS-mutant and wild-type cells, but not in H23 cells (Fig. 3D and E; Supplementary Fig. S3C and S3D). This difference is likely attributable to the lack of ATM activity in H23 cells (34, 35). These results suggest that radiosensitization by the combination of WEE1 and PARP inhibitors is associated with AZD1775-mediated DNA replication stress.

To test the hypothesis that AZD1775-mediated nucleotide depletion and subsequent replication stress are sufficient for radiosensitization in KRAS-mutant NSCLC cells, we next designed studies in which exogenous nucleosides were used to restore the nucleotide shortage. Although nucleosides had no effect on olaparib-mediated radiosensitization of Calu-6 cells, radiosensitization by AZD1775 was completely reversed by the addition of
nucleosides (Table 1B, Fig. 4A). Perhaps surprisingly, olaparib prevented the rescue of AZD1775-mediated radiosensitization by the addition of exogenous nucleosides. This result suggested that the interaction between WEE1 and PARP inhibition on radiosensitization is independent of nucleotide levels. In parallel studies, we examined the effect of nucleosides on pan-nuclear...
γH2AX staining, a marker of DNA replication stress (31, 32). Consistent with the high intensity γH2AX population observed by flow cytometry in response to AZD1775 treatment (Fig. 3D and E), AZD1775 alone or in combination with olaparib/radiation caused an increase in the percentage of cells with pan-nuclear γH2AX staining (Fig. 4B and C). The addition of exogenous nucleosides significantly reduced both the pan-nuclear and the high intensity γH2AX staining (Fig. 4B and C; Supplementary Fig. S4A and S4B), confirming that this staining pattern is a consequence of nucleotide depletion. Furthermore, the rescue of pan-nuclear γH2AX staining by nucleosides was not due to rescue of AZD1775-mediated inhibition of homologous recombination as RAD51 focus formation was not affected by the addition of nucleosides. (Supplementary Fig. S4C). Taken together, these data indicate that whereas nucleoside repletion is sufficient to rescue AZD1775-mediated replication stress and radiosensitization, the combination of AZD1775 and olaparib causes radiosensitization through a mechanism independent of AZD1775-mediated nucleotide depletion.

Given the importance of PARP1 trapping to the cytotoxic activity of PARP inhibitors, and its ability to impede DNA replication (17), we next investigated the contribution of PARP1 trapping to radiosensitization by AZD1775 and olaparib. PARP1 protein levels were measured in both chromatin fractions and whole-cell lysates of Calu-6 cells treated with radiation and olaparib or AZD1775. Consistent with inhibition of PARP catalytic activity, olaparib caused a reduction in PAR levels in both whole-cell lysates and chromatin fractions, while AZD1775 had no effect (Fig. 5A). Chromatin-associated PARP1 was increased in response to olaparib alone or the combination of olaparib with AZD1775, while AZD1775 alone did not increase chromatin-associated PARP1 levels (Fig. 5A and B). These results demonstrate that PARP1 trapping occurs under radiosensitizing conditions of AZD1775 and olaparib.
To begin to discern the relative contributions of PARP1 trapping and PARP catalytic inhibition to radiosensitization by combined WEE1 and PARP inhibition, we used two independent approaches. First, we used siRNA to deplete PARP1 from Calu-6 cells. PARP1 depletion should mimic the effects of PARP1 catalytic inhibition without PARP1 trapping. As expected, depletion of PARP1 protein resulted in an overall decrease in PARP catalytic activity, as assessed by PAR levels, similar to that achieved by olaparib (Fig. 5C) and treatment with PARP1 siRNA in the absence of AZD1775 produced modest radiosensitization (Table 1C; Fig. 5D). However, in contrast to the potentiation of AZD1775-mediated radiosensitization seen with olaparib, treatment with PARP1 siRNA in combination with AZD1775 did not further radiosensitize cells. This result suggests PARP1 trapping plays a role in radiosensitization by combined WEE1 and PARP inhibition.

To further distinguish the effects of PARP catalytic inhibition from PARP1 trapping on radiosensitization, we used veliparib, a PARP inhibitor with less potent PARP1 trapping activity than olaparib, at a concentration that did not trap PARP1 but inhibited PARP catalytic activity similarly to olaparib (1 μmol/L; Supplementary Fig. S5; refs. 16, 18, 36). Treatment with veliparib alone produced modest radiosensitization, but did not potentiate AZD1775-mediated radiosensitization (Table 1D; Fig. 5E). Taken together, the findings that PARP1 siRNA and veliparib can both produce modest radiosensitization in the absence of PARP1 trapping suggests that PARP catalytic inhibition is sufficient for radiosensitization. However, the finding that neither PARP1 siRNA nor veliparib radiosensitized when given in combination with AZD1775 suggests that inhibition of PARP catalytic activity is not sufficient to potentiate the radiosensitization that results from WEE1 inhibition. These findings are in contrast with the significant increase in radiosensitization caused by olaparib when given in combination with AZD1775 (Table 1A) and support the importance of PARP1 trapping as a mechanism of radiosensitization by the combination of WEE1 and PARP inhibitors.

We next sought to confirm the activity of WEE1 and PARP inhibition as a radiosensitizing strategy in vivo. Mice bearing Calu-6–derived xenografts were treated for 5 days with AZD1775.
olaparib, and radiation. Treatment with AZD1775 or olaparib in the absence of radiation did not significantly affect tumor growth as assessed by tumor volume doubling (Fig. 6; Supplementary Table S1). In combination with radiation however, AZD1775 or olaparib caused significant tumor growth inhibition associated with 6 and 5-day delays, respectively, in the tumor volume doubling time relative to radiation alone. Importantly, the combination of AZD1775 and olaparib caused an 11-day tumor growth delay relative to radiation alone and radiosensitization that was significantly greater than that achieved by either agent alone. Taken together, these results demonstrate the therapeutic efficacy of the combination of WEE1 and PARP inhibitors in vivo in KRAS-mutant NSCLC.

Discussion

In this study, we have found that the combination of WEE1 and PARP inhibition provides enhanced radiosensitization in KRAS-mutant lung cancers over and above the radiosensitization provided by either agent alone. Although single-agent PARP inhibitor can radiosensitize independently of PARP1 trapping, as exemplified by veliparib, for an enhanced radiosensitization effect in combination with WEE1 inhibitor, PARP1 trapping activity, such as that provided by olaparib, is required. This is almost certainly because trapped PARP1 has the potential to stall DNA replication fork progression and increase DNA replication stress. Moreover, unlike radiosensitization by single-agent AZD1775, the enhanced radiosensitization of KRAS-mutant NSCLC cells by the combination of AZD1775 and olaparib cannot be rescued by the addition of nucleosides. This suggests that the DNA replication stalling caused by PARP1 trapping in response to olaparib is dominant over the DNA replication stress that results from WEE1 inhibitor–induced nucleotide depletion. The fact that the combination of AZD1775 and olaparib provides greater radiosensitization than olaparib alone still demonstrates the importance of WEE1 inhibition for the enhanced activity though, presumably through one or more of the consequences of AZD1775-induced CDK1/2 activation such as increased origin firing.

Our focus on KRAS-mutant NSCLC is based in part on prior studies showing KRAS-mutant tumor cells have an increased reliance on DNA damage response pathways (2, 3). Furthermore, given the increased level of oncogene-induced replication stress in KRAS-mutant tumor cells, strategies that potentiate DNA replication stress may be especially effective. Although our data with the H1703 isogenic cell lines did not suggest a preferential radiosensitization by WEE1 and PARP inhibition in KRAS-wild-type cells, this is likely due to the high levels of underlying DNA replication stress even in the KRAS wild-type H1703 cells attributable to increased c-MYC expression and reduced ATM and CDKN2A expression (37). Nonetheless, the findings of this study do demonstrate that KRAS-mutant tumors can be effectively treated with this combination.

The contribution of DNA replication stress to the radiosensitization induced by WEE1 and PARP inhibition is likely related to an interaction between the unique functions of WEE1 and PARP in DNA replication. WEE1 inhibition causes CDK1/2 hyperactivation, leading to increased origin firing, nucleotide exhaustion, and impaired DNA elongation (6, 14), the latter two of which can be rescued by exogenous nucleosides. In response to DNA replication stress, PARP inhibition impairs the stabilization and restart of stalled DNA replication forks (11, 12), events that are likely nucleotide independent. These effects represent a potential mechanism whereby WEE1 and PARP inhibition may interact to impair DNA replication at multiple points in the DNA replication process. One hypothesis is that increasing origin firing caused by AZD1775 potentiates the effects of trapped PARP on DNA replication by increasing the probability of ongoing DNA replication forks colliding with trapped PARP. This concept is supported by our findings that nucleosides rescue AZD1775 radiosensitization but not sensitization from the combination of AZD1775 and olaparib, suggesting that olaparib acts independent of nucleotide levels in the DNA replication process. Although PARP inhibition is known to prevent recovery of stalled DNA replication forks (11), whether or not PARP inhibitors cause fork stalling is unknown. Further studies are needed to better understand the interaction between radiation and PARP inhibitors on the DNA replication process.

Although other studies have attributed the monotherapy (16) and chemosensitizing (18, 19) activity of PARP inhibitors to PARP1 trapping, the relative roles of PARP catalytic inhibition and PARP1 trapping to radiosensitization have not been previously investigated. Radiation creates a diverse array of DNA damage, including oxidized bases, SSBs (from BER intermediates or frank SSBs), and DSBs. Although it is not clear which of these lesions might serve as the substrate for PARP (15), our data clearly demonstrate the binding of PARP1 to chromatin in response to radiation-induced DNA damage and olaparib treatment. PARP1 trapping, however, was not required for olaparib-mediated
Radiosensitization, a finding consistent with the ability of veliparib to radiosensitize under conditions, which should not trap PARP (38). In contrast, the importance of PARP1 trapping to radiosensitization by combined AZD1775 and olaparib is supported by the finding that PARP catalytic inhibition alone is insufficient to potentiate AZD1775-mediated radiosensitization. Given that PARP inhibitors vary in their PARP1 trapping potency, these mechanistic studies can inform the appropriate choice of PARP inhibitor for combination therapeutic strategies.

As the majority of locally advanced cancers are treated with a combination of concurrent radiation and chemotherapy, it is important to develop the cytotoxic activity of WEEl and PARP inhibitors in the absence of radiation. Ongoing clinical trials will define the dose and schedule of the combination of AZD1775 and olaparib in the metastatic setting (NCT02511795). These studies will likely lead to trials evaluating this combination with radiation in locally advanced cancers. On the basis of the outcome of these studies, future trials may substitute novel combinations of targeted agents with cytotoxic systemic activity for chemotherapy in chemoradiation regimes, a strategy that has the promise to be effective and potentially less toxic than standard cytotoxic chemotherapy.

Disclosure of Potential Conflicts of Interest

M.A. Morgan reports receiving a commercial research grant from and has received speakers bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

References


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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.A. Parsels, D. Karnak, J.D. Parsels, Q. Zhang, J. Maybaum
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Other (participated as a summer research fellow and made some initial trials with the cells and collected data): J. Velez-Padilla

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