PARP inhibition selectively increases sensitivity to cisplatin in ERCC1-low non-small cell lung cancer cells

Haiying Cheng1,2, Zhenfeng Zhang1, Alain Borczuk3, Charles A. Powell4, Adayabalam S. Balaje4, Howard B. Lieberman5,6 and Balazs Halmos1,*

1Department of Medicine, Division of Hematology/Oncology, Herbert Irving Comprehensive Cancer Center, New York Presbyterian Hospital-Columbia University Medical Center, New York, NY 10032, USA, 2Department of Medicine, Division of Medical Oncology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY 10461, USA, 3Department of Pathology, New York Presbyterian-Hospital-Columbia University Medical Center, New York, NY 10032, USA, 4Department of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA, 5Center for Radiological Research, Department of Radiation Oncology, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA and 6Department of Environmental Health sciences, Mailman School of Public Health, Columbia University Medical Center, New York, NY 10032, USA

To whom correspondence should be addressed. Balazs Halmos, Division of Hematology/Oncology, Herbert Irving Comprehensive Cancer Center, New York Presbyterian-Hospital-Columbia University Medical Center, New York, NY, USA. Tel: +212-851-4637; Fax: +212-851-4774; Email: bh2376@columbia.edu

Platinum compounds are the foundation of chemotherapy regimens for non-small cell lung cancer (NSCLC) despite poor response rates and limited response duration. It has been reported that tumor expression of excision repair cross-complementation group 1 (ERCC1), a key component in nucleotide excision repair, may correlate with clinical response to platinum agents. We found that most primary lung tumor specimens demonstrated a stronger protein expression of poly (adenosine diphosphate ribose) polymerases 1 (PARP1) than their normal counterparts. Therefore, we hypothesized that combining PARP inhibition with platinum compounds may be an approach to improve platinum-based therapy for NSCLC. Drug combination experiments revealed that two distinct PARP inhibitors, olaparib and veliparib, not only potentiated the cell killing by cisplatin but also conferred cytotoxicity as a single agent specifically in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 lung cancer cells. Moreover, small interfering RNA knockdown of ERCC1 in A549 and H157 cells increased their sensitivities to both cisplatin and olaparib in a synergistic manner in our model. Furthermore, mechanistic studies indicated that combined PARP inhibitor and cisplatin could lead to sustained DNA double-strand breaks, prolonged G2/M cell cycle arrest with distinct activation of checkpoint kinase 1 signaling and more pronounced apoptosis preferentially in lung cancer cells with low ERCC1 expression. Collectively, these data suggest that there is a synergistic relationship between PARP inhibition and low ERCC1 expression in NSCLC that could be exploited for novel therapeutic approaches in lung cancer therapy based on tumor ERCC1 expression.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Despite recent advances in treatment, the 5 year survival rate is dismal at around 15%. Most effective chemotherapy regimens for advanced lung cancer remain platinum based and lead to response rates of 15–30% in unselected patients and a median survival of 10–12 months (1). Primary and acquired resistance to platinum agents is a serious clinical problem. Its mechanisms are probably multifactorial and remain poorly understood. Platinum agents generally function by forming platinum-DNA adducts, disrupting DNA structure through cross-links and subsequently resulting in cell death. Enhanced DNA repair can lead to increased cell viability in the face of DNA damage and has been proposed to be important in mediating platinum resistance (2).

Platinum–DNA adducts are removed mainly by the nucleotide excision repair (NER) pathway (3). The excision repair cross-complementation group 1 (ERCC1) protein is a key component of NER and is required to incise the damaged DNA. ERCC1 is also involved in interstrand cross-link (ICL) repair, double-strand break (DSB) repair, homologous recombination and telomere maintenance (4). Several lines of evidence have implicated differential ERCC1 expression in mediating cellular resistance to platinum. For instance, suppression of ERCC1 levels increased the sensitivities to cisplatin in platinum-resistant ovarian cancer cell lines (5), whereas forced expression of ERCC1 in ERCC1-deficient Chinese hamster ovary cells led to platinum resistance (6). When evaluated in clinical trials, the International Adjuvant Lung Cancer Trial demonstrated that only ERCC1 low/negative but not ERCC1 high/positive tumors had a survival benefit from cisplatin-based adjuvant chemotherapy (7). One prospective, randomized study assigned patients in the control arm to non-selective chemotherapy while in the other arm patients with low tumor ERCC1 messenger RNA (mRNA) levels received a platinum-containing and patients with high tumor ERCC1 mRNA received a non-platinum containing regimen. ERCC1 level directed treatment lead to a significant improvement in response rates as compared to non-selective chemotherapy (50.7% in the ERCC1 biomarker-directed arm versus 39.3% in the control arm) (8). In addition, a meta-analysis of 12 studies (836 patients) in advanced lung cancer showed a higher response rate to platinum regimens in patients with low ERCC1 expression as compared with patients with high ERCC1 expression (47% versus 28%) (9). Interestingly, a recent report using specimens from the International Adjuvant Lung Cancer Trial-Bio study further indicated that the predictive role of ERCC1 on cisplatin benefits may correlate with the histological subtype of lung cancers such that ERCC1 displayed significant predictive power in squamous cell carcinoma but not in adenocarcinoma in that study (10). Taken together, these data suggest that tumor expression of ERCC1 may correlate with clinical response to platinum agents, but the limited benefit of platinum drugs even in ERCC1-low-expressing tumors remains a substantial issue.

DNA repair pathways other than NER may also contribute to platinum resistance. Inhibition of poly (adenosine diphosphate ribose) polymerases (PARPs), key components of base excision repair, have been shown to enhance platinum sensitivity in BRCA-associated breast and ovarian cancers (11,12). Inhibition of PARPs, which are essential for repairing DNA single-strand breaks, can lead to an accumulation of more lethal DNA DSBs. PARP1 constitutes >80% of overall PARP activity and has been identified as a platinum–DNA damage response protein (13). Preclinical data suggested that PARP inhibitors synergized with platinum in BRCA1/2-deficient mammary tumor cells (14–16). Phase II clinical trials further demonstrated the efficacy and safety of olaparib in BRCA-associated breast and ovarian cancers (17,18). Moreover, a recent randomized phase II study demonstrated that maintenance therapy with olaparib could provide a significant improvement in progression-free survival in patients with platinum-sensitive relapsed serous ovarian cancer (19). The antitumor activity of PARP inhibition in BRCA-associated breast/ovarian cancers provides a clinical application of the concept of synthetic lethality (20–22).
In this study, we hypothesized that the combined impairment of two complimentary DNA repair pathways (PARP inhibition and low ERCC1 expression) would synergistically increase the sensitivity to platinum agents in lung cancers.

Materials and methods

Cell lines and material

The following non-small cell lung cancer (NSCLC) cell lines were obtained from American Type Tissue Collection (Manassas, VA): HCC827, H23, H157, H441, A549 and NHBE (normal human bronchial epithelial cells). PC9 cells were a gift from Dr Susumu Kobayashi (Harvard Medical School, Boston, MA). Lung cancer cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic/antimycotic (Invitrogen, Carlsbad, CA) and were in the logarithmic growth phase at the initiation of all experiments. The PARP inhibitors, olaparib (AZD2281) and veliparib (ABT-888), were obtained from Selleck Chemicals (Houston, TX); Cisplatin was obtained from Sigma–Aldrich (St Louis, MO).

Immunoblotting and antibodies

Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) for western blot analysis as described previously (23). Antibodies against ERCC1 (FL-297), RAD51 (H-92), PARP1 (N-20), CHK1 (G-4) and CHK2 (H-300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP, cleaved PARP (Asp214), P53 (F-1) and PARP1 (N-20) were obtained from Cell Signaling Technology (Boston, MA). Anti-PARP rabbit polyclonal antibody was obtained from Trevigen (Gaithersburg, MD). Antibody against phospho-histone H2A.X (Ser 139) was purchased from Millipore (Temecula, CA). The protein amounts were analyzed semi-quantitatively by assessing band intensity with the use of Quantity One software (Bio-Rad).

Immunohistochemistry

As described previously (23), formalin-fixed primary lung tumor tissue sections were deparaffinized and rehydrated and incubated with 0.6% hydrogen peroxide in methanol, followed by staining using the ready-to-use Vectastain Universal Quick Kit (Vector Laboratories, Burlingame, CA). Antigen retrieval treatment with sodium citrate (10 mM, pH 6.0) was used for the detection of PARP1. Antibodies were used at a dilution of 1:100 (optimal dilution for overnight incubation) at 4°C followed by hematoxylin nuclear counterstaining. Two negative controls were used for PARP1 expression: (i) control including blocking peptide for PARP1 (Santa Cruz Biotechnology) and (ii) control without incubation with PARP1 antibody. PARP1 expression in lymphocytes was used as internal positive control.

Drug combination studies with clonogenic survival assay

Clonogenic survival was assessed as described previously (24). Logarithmically growing cells were plated in triplicate in six-well tissue culture dishes containing media alone or media supplemented with drugs. Cisplatin and olaparib were added for 3 days, whereas veliparib was present for the entire duration of the experiment as described previously (25). After 3–14 days, colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. Surviving colonies were defined as colonies containing >50 cells. Survival was expressed as the relative plating efficiency as compared with control cells. The Bliss model was used to calculate the combination index (CI) and to evaluate the effects of drug combinations (26). F1, f2 and f12 represents the effects from single drug 1, single drug 2 and the combination of drugs 1 and 2. The Bliss CI (CIbliss) = (f1 + f2 – f1^2/2)/f2. The drug–drug interactions were defined as synergism if CI < 0.99, antagonism if CI > 1.01, otherwise additive. CIs were determined using the MTS Assay Kit according to the manufacturer’s instructions (Thermo). Levels of ERCC1 knockdown at different time points were assessed by immunoblot analysis from pools of transfected cells.

Quantitative reverse transcription–PCR assay

As described previously (28), total RNAs were collected using RNeasy Mini Kit (QiaGen, Germantown, MD) from cells. Complementary DNAs were synthesized with Moloney marine leukemia virus reverse transcriptase (SuperScript III reverse transcriptase) with the use of oligo(denethylminated) primers from Invitrogen. All samples were run in triplicates on Roche Light Cycler with the use of Syber green probes (Roche Applied Sciences, Indianapolis, IN) using the following variables: denaturation at 95°C for 10 min, followed by 45 cycles of amplification (95°C for 10 s, 60°C for 10 s and 72°C for 15 s) and cooled to 4°C at a rate of 20°C per second. Levels of glyceraldehyde-3-phosphate dehydrogenase and β-actin expression were used as internal reference to normalize input complementary DNA. Ratios of level of each gene to glyceraldehyde-3-phosphate dehydrogenase were then calculated. Primer sequences are as follows: ERCC1 F1, 5’-TGTTGCGGTTGGATGA-3’; ERCC1 F2, 5’-ctgttggtcatacagg-3’. Statistics

All data are expressed as mean ± SD from at least triplicates. Statistical analysis was performed by one- or two-way analysis of variance, as appropriate using Statistica 6.0 (StatSoft, Tulsa, OK). Differences were considered significant at P < 0.05.

Results

Expression of PARP and ERCC1 in NSCLC

As shown in Figure 1A, HCC827, PC9 and H441 lung cancer cells have relatively lower ERCC1 expression in comparison with NHBE cells, whereas H157, A549 and H23 lung cancer cells have higher ERCC1 protein expression with levels similar to NHBE cells, as determined by western blotting. Similar results were observed when ERCC1 mRNA levels in different cell lines were determined by quantitative reverse transcription–PCR (Figure 1C). These results are consistent with variable ERCC1 expression levels reported in lung tumors.

Next, we determined PARP1 expression by immunohistochemistry in 12 formalin-fixed, paraffin-embedded pre-therapeutic specimens from patients with NSCLC (Figure 1B). PARP1 was found to be expressed in every specimen with variable intensities and mainly as a nuclear protein (Table 1). Overall, the expression was stronger in tumor cells compared with stromal cells or airway epithelial cells in the majority (11 of 12) of specimens. The protein expression of PARP1 in NSCLC cell lines were determined by western blot (Figure 1A). These results suggest fairly ubiquitous and strong expression of PARP in lung cancer cells.

PARP inhibition selectively synergized with cisplatin in ERCC1-low NSCLC cells

To assess cellular toxicity for different concentration-combinations of cisplatin (1, 3 and 10 μM) and olaparib (PARP inhibitor: 1, 3 and 10 μM), clonogenic survival assays were performed (Figure 2A). Olaparib selectively augmented cisplatin-induced cytotoxicity in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells. When the Bliss model was further used to determine the combination index and drug–drug interactions (CI < 0.99, synergism, CI > 1.01, antagonism, otherwise additive), it was discovered that olaparib selectively synergized with cisplatin only in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells. Effects on cell viability by different concentration-combinations of

740
cisplatin and olaparib (PARP inhibitor) were also determined by MTS cell growth assays (data not shown). Analogous to the results of clonogenic assays, olaparib specifically improved cisplatin sensitivity in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells. In addition, as a single agent, olaparib conferred selective cytotoxicity against ERCC1-low HCC827 and PC9 cells but had no effects by itself in ERCC1-high cells.

In addition to olaparib, an alternative PARP1/2 inhibitor, veliparib (ABT-888) (16) was also examined. Veliparib has been used at doses of 10 mg orally, daily (29) to 200 mg twice daily depending on the concurrent chemotherapy in phase I trials. However, it remains unclear which type of schedule is optimal for veliparib activity, for example, prolonged exposure versus pulse exposure. Therefore, we first performed clonogenic survival assays to determine the type of veliparib exposure that would most effectively increase cisplatin sensitivity in HCC827 cells. It was found that the most significant cytotoxic effects were observed when cells were first simultaneously incubated with both cisplatin and veliparib followed by continuous veliparib exposure after removal of cisplatin (schedule VI in Figure 2B). Therefore, subsequent clonogenic survival assays were performed by continuous exposure to veliparib. Similar to the effects of olaparib, veliparib preferentially increased the cytotoxicity of cisplatin in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells (Figure 2C). Furthermore, if olaparib was given using the same treatment schedule in clonogenic assays as veliparib, even stronger cytotoxicity was observed in cells with continuous incubation of olaparib (Figure 2D). Taken together, two different PARP inhibitors augmented cisplatin sensitivities selectively in ERCC1-low lung cancer cells illustrating target specific effects.

**ERCC1 knockdown sensitized cells to cisplatin and olaparib**

Studies have shown that ERCC1 knockdown could potentiate platinum sensitivities in some cancer cell lines (5) and we hypothesized that ERCC1 knockdown could also enhance the efficacy of cisplatin in ERCC1-high NSCLC cells. Therefore, we next examined the role of ERCC1 knockdown on sensitizing NSCLC cell lines to cisplatin and PARP inhibition. As demonstrated in Figure 3A, transfection of specific siRNA targeting ERCC1 in ERCC1-high A549 cells and H157 cells significantly inhibited ERCC1 protein expression, whereas the non-targeted siRNA or vehicle alone had no effects. In addition, knockdown of ERCC1 significantly increased not only cisplatin sensitivity but also olaparib sensitivity in both A549 and H157 cells (Figure 3B and C) ($P < 0.05$). Furthermore, additive or synergistic effects between cisplatin and olaparib were observed when ERCC1 expression was suppressed in this model. For example, there were no synergistic effects between 1 µM cisplatin and 1 µM olaparib in A549 cells treated with non-targeted siRNA. However, when ERCC1 expression was inhibited by ERCC1 siRNA, there were more pronounced cytotoxicity for the combined 1 µM cisplatin and 1 µM olaparib with CI < 1 and thus synergistic effects were noted between cisplatin and olaparib under these circumstances. Similar results were noticed when ERCC1 knockdown was obtained with ERCC1 siRNA pools (data not shown).
A

**ERCC1-low HCC827**

Surviving fraction

- Control
- 1μM Olaparib
- 3μM Olaparib
- 10μM Olaparib

**ERCC1-low PC9**

Surviving fraction

- Control
- 1μM Olaparib
- 3μM Olaparib
- 10μM Olaparib

B

<table>
<thead>
<tr>
<th></th>
<th>d1</th>
<th>d2</th>
<th>d3</th>
<th>d4</th>
<th>d5</th>
<th>d6</th>
<th>d7</th>
<th>d8</th>
<th>d9</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1C</td>
<td>1C</td>
<td>1C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1C + 10V</td>
<td>1C + 10V</td>
<td>1C + 10V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1C + 10V</td>
<td>1C + 10V</td>
<td>10V</td>
<td>10V</td>
<td>10V</td>
<td>10V</td>
<td>10V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1C + 50V</td>
<td>1C + 50V</td>
<td>10V</td>
<td>10V</td>
<td>10V</td>
<td>10V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>1C + 50V</td>
<td>1C + 50V</td>
<td>1C + 50V</td>
<td>50V</td>
<td>50V</td>
<td>50V</td>
<td>50V</td>
<td>50V</td>
<td></td>
</tr>
</tbody>
</table>

**Surviving Fraction (%)**

- 0
- 0.2
- 0.4
- 0.6
- 0.8
- 1

- 50V
- 10V
- 1C
PARP inhibition in lung cancer

Fig. 2. (A) Clonogenic survival assays were performed to assess cellular toxicity for different concentration-combinations of cisplatin (1, 3 and 10 µM) and olaparib (PARP inhibitor: 1, 3 and 10 µM). Olaparib selectively augmented cisplatin-induced cytotoxicity in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells. (B) HCC827 cells were treated with veliparib and cisplatin using the exposure schemes. As determined by clonogenic survival assays (Figure 3B), the most significant cytotoxic effects were observed when cells were first simultaneously incubated with both cisplatin and 50 µM veliparib and then with continuous veliparib exposure after removal of cisplatin. (C) Cellular toxicity for different concentration-combinations of veliparib (ABT-888: 5, 10 and 50 µM) and cisplatin (1, 3 and 10 µM) was examined in clonogenic survival assays. Veliparib preferentially increased cytotoxicity of cisplatin in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 cells. Combination index values for PARP inhibitors (olaparib or veliparib) and cisplatin were calculated. The drug–drug interactions were defined as synergism if CI < 0.99, antagonism if CI > 1.01, otherwise additive. ‘∆∆’ indicates CI ≤ 0.70, ‘∆’ indicates 0.70 < CI < 0.99. Except for combinations labeled with ‘∆∆’ or ‘∆,’ CI = 1.00 for the rest of drug combinations in HCC827 and PC9 cells. The majority of CIs were >1.01 in A549 and H157 cells. (D) Olaparib was given using the same treatment schedule in clonogenic assays as veliparib and stronger cytotoxicity was observed in cells with continuous incubation of olaparib. 0.3P = 0.3 µM olaparib, 1P = 1 µM olaparib.
Effects of cisplatin and olaparib on induction of PAR formation, γ-H2AX and apoptosis

Formation of the PAR polymers is an indicator of PARP activity. PAR formation in cells was measured following 24, 48 and 72 h treatment of cisplatin and olaparib. As shown in Figure 4A, in ERCC1-low HCC327 cells, the PAR levels were significantly increased following cisplatin treatment and were markedly suppressed with the PARP inhibitor, olaparib. Similar trends were observed in ERCC1-high A549 cells (Figure 4B) demonstrating effective target inhibition despite no synergy noted in these ERCC1-high cells.

To gain insights into the molecular mechanisms on how the synergism between PARP inhibition and ERCC1 loss could improve cisplatin sensitivity, the cells were first treated with 3 µM cisplatin +/- 3 µM olaparib for 1 day, then the dynamic effects of cisplatin and olaparib on DNA damage, DNA repair and apoptosis were determined by western blot after withdrawal of the drug treatment for 0, 24, 48 and 72 h.
Induction of γ-H2AX and RAD51 is considered as markers for DNA DSBs and homologous recombination repair, respectively. Retention of γ-H2AX can also be caused by stalled replication (30). As indicated in Figure 5A, there was persistent γ-H2AX and RAD51 formation after withdrawing the combined treatment with cisplatin and olaparib in ERCC1-low-expressing HCC827 but not in ERCC1-high-expressing A549 cells. In corroboration, increased apoptotic death indicated by cleaved PARP was also observed after withdrawing combined cisplatin and olaparib in ERCC1-low-expressing HCC827 but not in ERCC1-high-expressing A549 cells. Similar experiments were further performed in ERCC1 suppressed A549 and H157 cells by transfection of ERCC1 siRNA. As indicated in Figure 5B, ERCC1 knockdown in A549 and H157 cells led to markedly increased γH2AX formation and apoptosis (cleaved PARP) following withdrawal of cisplatin and olaparib indicating that the effectiveness of cisplatin and olaparib combined treatment depends on the expression level of ERCC1. Collectively, our data suggest that the high levels of DNA damage caused by cisplatin and olaparib may saturate the limited DNA repair capacity in cells with low ERCC1 expression, leading to persistent stalled DNA replication forks and DSBs, and subsequently activation of a signaling cascade to induce apoptosis. On the other hand, high expression level of ERCC1 may ensure an efficient repair of cisplatin-induced DNA ICLs despite the appreciable level of PARP inhibition.

Effects of cisplatin and olaparib on G2/M cell cycle arrest and checkpoint activation

Cisplatin can induce stalled replication forks and DNA DSBs, which subsequently activate the cell cycle checkpoint pathways (30). Prolonged G2 arrest was previously proposed as an essential process in mediating cisplatin-related cell death (31). To investigate the effects of PARP inhibition and cisplatin on cell cycle control, BrdU cell proliferation assays were carried out 72h after treatment with cisplatin and/or olaparib. As shown in Figure 6B, there was a significant increase in the G2/M population in ERCC1-low HCC827 and PC9 cells by either cisplatin or olaparib. The combination of cisplatin and olaparib led to an even more prominent G2/M arrest than either agent alone. In contrast, no marked G2/M arrests were observed in ERCC1-high A549 and H157 cells following the same treatment. To investigate whether there were similar trends in G2/M arrest at an earlier time point, cell cycle analysis was performed after 24h of drug treatment. It was found that 24h incubation of cisplatin led to significant G2/M arrest in all the four cell lines regardless of ERCC1 expression status, whereas olaparib only caused G2/M arrest in ERCC1-low HCC827 and PC9 cells (data not shown).

We then evaluated the activation of checkpoint kinases, checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2), which are crucial in controlling G2/M and G1/S transitions (32). Twenty-four hour treatment with cisplatin and olaparib either alone or in combination increased phospho-CHK1 (S345) level in ERCC1-low HCC827 cells, but not in ERCC1-high A549 cells (Figure 6C). However, under the same circumstances, phosphorylation of CHK2 (T68) in both HCC827 and A549 cells was unchanged. Moreover, cisplatin and olaparib either alone or in combination increased phospho-p53 (S15) similarly in both ERCC1-low HCC827 cells and ERCC1-high A549 cells. P53 regulates both pro-apoptotic and antiapoptotic signaling and thus plays a dual role: there are reports that it can either sensitize or cause resistance to cisplatin (30). Regardless, under our experimental conditions, there was differential activation of CHK1 but not CHK2 or p53 in ERCC1-high and ERCC1-low cells suggestive of a key role of CHK1 activation in the functional sequelae of drug treatment in ERCC1-low cells.

Discussion

Platinum compounds are the foundation of chemotherapy regimens for NSCLC but nevertheless response rates are disappointing and response duration is limited due to primary and acquired platinum resistance. ERCC1 removes platinum-induced DNA damage and accordingly ERCC1 expression is a candidate biomarker to predict platinum sensitivity. We hypothesized that inhibition of PARP-associated pathway(s) could enhance the effectiveness of platinum therapy. Therefore, in this study, we explored whether combining PARP inhibition with platinum compounds would be an approach to improve platinum-based therapy for NSCLC. Additionally, we investigated whether the effectiveness of this approach could be modulated by ERCC1 expression. We first determined that PARP1 protein was expressed in all of 12 primary lung tumor specimens and in
NSCLC cell lines that we examined because one concern from clinical trials utilizing PARP inhibitors in breast cancer is that lack of PARP expression in some breast cancers might have affected interpretation of results (33). We then showed that two distinct PARP inhibitors, olaparib and veliparib, selectively improved cisplatin activity in ERCC1-low HCC827 and PC9 but not in ERCC1-high A549 and H157 lung cancer cells. The ERCC1-low HCC827 and PC9 cells both harbor activating epidermal growth factor receptor mutations, and in concert with our findings, it has been reported that lung cancers with epidermal growth factor receptor activating mutations are more probably to be associated with low ERCC1 levels (34). Combination index analyses further corroborated that PARP inhibitors synergized with cisplatin in both ERCC1-low HCC827 and PC9 cells. Additionally, in agreement with previous reports (35), ERCC1 depletion led to increased sensitivity to cisplatin. Interestingly, knockdown of ERCC1 also potentiated cell killing effects of PARP inhibition alone and in combination with cisplatin. These data suggest that there may be a synthetically lethal relationship between PARP inhibition and ERCC1 loss in NSCLC. Previous studies, including RNA interference screens, have identified some genes that when attenuated may improve the sensitivity of PARP inhibitor therapy. Most of those genes have authentic roles in DNA damage response, such as CDK1, CHK1, CHK2, BRCA1, ATR, ATM, RAD51, MRE11, PTEN, XRCC1, DDB1 and XAB2 (25,36–39). Intriguingly, some of the listed genes are involved in DNA repair pathways besides homologous recombination. Our study suggests that ERCC1 loss can also sensitize tumor cells to PARP inhibition.

To determine the mechanisms underlying the synergy between PARP inhibition and ERCC1 loss, we next investigated the dynamic effects of olaparib and cisplatin on PARP activity, DNA damage, DNA repair and apoptosis. We found that olaparib significantly blocked cisplatin-related PAR induction in both ERCC1-low and ERCC1-high cells. However, combined treatment of cisplatin and olaparib only led to persistent γ-H2AX formation (marker of DNA DSBs) and more pronounced apoptotic death in lung cancer cells with low ERCC1 expression. Emerging evidence indicates that ERCC1 may engage in diverse DNA repair mechanisms. Besides its well-described role in NER, it also has been reported to function in ICL repair, DSB repair and homologous recombination (4, 40). For instance, ERCC1 was found to be essential for the completion of homologous recombination involved in repairing DNA DSBs caused by ICLs at a step downstream of RAD51 foci formation (41). In accordance with previous findings, our data support the role of ERCC1 in the reinitiation and repair of cisplatin-induced stalled replication forks and DNA DSBs. Thus, the synergism between PARP inhibition and low ERCC1 expression could at least partly be attributed to the roles of ERCC1 in homologous recombination repair and DSB repair. On the other hand, in addition to its role in DNA single-strand break repair, PARP1 is also activated at stalled replication forks and plays an important role in restarting stalled replication forks (42,43). Thus, PARP inhibition could block the restarting of cisplatin-induced stalled replication forks, thereby possibly causing sustained DNA damage and subsequently potentiating the cytotoxicity of cisplatin in cells with low DNA repair capacity.
PARP inhibition in lung cancer

A

B

ERCC1-low HCC827

72-hour drug treatment

ERCC1-low HCC827

ERCC1-low PC9

ERCC1-high A549

ERCC1-high H157

Downloaded from https://academic.oup.com/carcin/article-abstract/34/4/739/2463029
by guest
on 20 August 2018
It is known that cisplatin-induced DNA damage can trigger G₂/M cell cycle arrest through activation of checkpoint signaling (30). Therefore, we investigated the effects of olaparib and cisplatin on cell cycle regulation and checkpoint activation. We found that PARP inhibition alone and in combination with cisplatin led to prolonged G₂/M arrest selectively in ERCC1-low but not in ERCC1-high NSCLC cells and these findings interestingly correlate with their differential sensitivities to PARP inhibition and cisplatin. In line with our observations, a recent report suggested an essential role for ERCC1 in mitotic progression (44). We then examined the ATR-CHK1 and ATM-CHK2 pathways, which are two principal mechanisms activated by DNA damage and their activation is involved in regulating cell cycle arrest in late G₂, intra-S and G₂₃ phases (32). We noticed distinct activation of checkpoint kinase 1 signaling but similar activity of checkpoint kinase 2 and p53 (serine 15) in NSCLC cells with differential ERCC1 expression. Taken together, cisplatin and olaparib selectively induced prolonged G₂/M arrest in ERCC1-low lung cancer cells, probably through activation of checkpoint kinase 1 signaling. Although there was no difference in early G₂/M arrest between ERCC1-low and ERCC1-high cells, persistence of G₂/M arrest up to 72 h in ERCC1-low cell lines was probably due to unrepairred DNA lesions that led to a sustained activation of cell cycle checkpoint signaling. Consistent with our cell cycle data, a recent study has demonstrated that the cellular sensitivity to cisplatin was due to delayed DNA damage processing and cell cycle control rather than damage response during the early times (45). Furthermore, an earlier study showed that induction of DNA ICL induced by 5 h treatment with cisplatin peaked between 3 and 8 h after the drug treatment, which declined to the basal level of mock treated cells by 24 h (46). The kinetics of ICL repair reported in this study correlates with the duration of G₂/M arrest we detected during the first 24 h of cisplatin treatment in ERCC1-high cell lines but not after 72 h time point at which the lesions would have been efficiently removed. In contrast, G₂/M arrest was still detectable in ERCC1-low cell lines even at 72 h probably owing to the persistence of DNA lesions.

Resistance to cisplatin and PARP inhibition is probably multifacto-
rrial and mechanisms other than differential ERCC1 expression might play key roles in certain cell types. Therefore, the synergistic effect of cisplatin and PARP inhibitors reported in this study may not be the same for all the lung tumor cells. In particular, BRCA1 expression has also been indicated as a predictive biomarker for platinum compounds and PARP inhibition in NSCLC (47–49). Therefore, future mechanis-
tic studies are certainly needed to demonstrate how exactly different DNA repair pathways are involved in the improved cytotoxicity of combining PARP inhibition with cisplatin in a wider range of lung cancers. High-throughput RNA interference screens are currently underway to further delineate the key pathway components sensitiz-
ing NSCLC to cisplatin and PARP inhibition. Nevertheless, our study
is the first so far implicating the potential of a synthetically lethal relationship between PARP inhibition and ERCC1 loss in NSCLC and suggests that drugs that can block ERCC1 function could have clinical potential in treating patients with lung cancer.

In summary, we have found that PARP inhibition selectively synergizes with cisplatin in ERCC1-low but not in ERCC1-high lung cancer cells. The synergism between PARP inhibition and ERCC1 loss could be exploited as a plausible strategy to enhance platinum therapeutic efficacy or even to utilize PARP inhibitor therapy alone in ERCC1-low lung cancers.

Funding
American Cancer Society (RSG-08-303-01 to B.H.;) the Pfizer Fellowships in Oncology (PG287897 to B.H. and H.C.;) Paul Calabresi Career Development Award for Clinical Oncology (5K12CA132783-04 to H.C.) and National Institutes of Health (GM079107 and CA130536 to H.B.L.).

Conflict of Interest Statement: None declared.

References