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Identification and characterization of synthetic viability with ERCC1 deficiency in response to interstrand crosslinks in lung cancer

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Abstract

Purpose: ERCC1/XPF is a DNA endonuclease with variable expression in primary tumor specimens, and has been investigated as a predictive biomarker for efficacy of platinum-based chemotherapy. The failure of clinical trials utilizing ERCC1 expression to predict response to platinum-based chemotherapy suggests additional mechanisms underlying the basic biology of ERCC1 in the response to interstrand crosslinks (ICLs) remain unknown. We aimed to characterize a panel of ERCC1 knockout () cell lines where we identified a synthetic viable phenotype in response to ICLs with ERCC1 deficiency.

Experimental Design: We utilized the CRISPR-Cas9 system to create a panel of ERCC1 lung cancer cell lines which we characterized.

Results: We observe that loss of ERCC1 hypersensitizes cells to cisplatin when wildtype (WT) p53 is retained, while there is only modest sensitivity in cell lines that are p53^{mutant/null}. Additionally, when p53 is disrupted by CRISPR-Cas9 (p53*) in ERCC1 /p53^{WT} cells, there is reduced apoptosis and increased viability after platinum treatment. These results were recapitulated in two patient data sets utilizing p53 mutation analysis and ERCC1 expression to assess Overall Survival. We also show that kinetics of ICL- repair (ICL-R) differ between ERCC1 /p53^{WT} and ERCC1 /p53* cells. Finally, we provide evidence that cisplatin tolerance in the context of ERCC1 deficiency relies on DNA-PKcs and BRCA1 function.

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AUTHOR CONTRIBUTIONS

SMP and GB conceived the project. JRH, WL, HZ conducted the biological experiments included in this manuscript. DW and AGS assisted with the lung adenocarcinoma patient analysis. JBB assisted with analysis of flow cytometry data. WC assisted with statistical analyses. JRH wrote the manuscript. SMP oversaw the project.

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Conclusions: Our findings implicate p53 as a potential confounding variable in clinical assessments of ERCC1 as a platinum biomarker via promoting an environment in which error-prone mechanisms of ICL- repair may be able to partially compensate for loss of ERCC1.

Keywords

ERCC1-XPF; cisplatin; DNA repair; interstrand crosslinks; nucleotide excision repair; homologous recombination

INTRODUCTION

The structure-specific endonuclease ERCC1 (Excision Repair Cross-Complementation Group 1)/XPF (Xeroderma Pigmentosum Group F) plays key roles in Nucleotide Excision Repair (NER), Interstrand Crosslink Repair (ICL-R), Homologous Recombination (HR) Repair and Single Strand Annealing pathways. While the role of ERCC1/XPF in NER is well established, the totality of its specific functions in the processing and repair of interstrand crosslinks (ICLs) has remained unclear (See (1, 2) for review). ICLs are produced upon exposure to agents that covalently link bases in opposing strands of DNA, and endonucleases are required for cleavage of the phosphodiester backbone adjacent to ICLs in order to initiate repair (3). Much recent evidence indicates ERCC1/XPF is required for ICL-unhooking, while other work highlights additional roles for this complex in ICL-R downstream of unhooking (4–7).

Use of interstrand crosslinking agents, including cisplatin, remain a mainstay in the treatment of malignancies. Several mechanisms for resistance to platinum-based chemotherapy have been described, including loss of Base Excision Repair and MMR, decreased drug accumulation, increased sequestering by thiols, decreased apoptosis, and increased translesion synthesis (8–11). Another proposed mechanism of resistance to cisplatin involves increased expression of ERCC1/XPF observed both *in vitro* with cisplatin-sensitive/resistant cell lines and in relation to survival in patient samples (11–17). Work by our laboratory and others have shown that down-regulation of ERCC1/XPF sensitizes cancer cells to cisplatin and that this sensitivity is related to a reduction in ICL and intrastrand adduct (ISA) repair (18). Additionally, small molecule inhibitors of ERCC1/XPF can increase cisplatin sensitivity both *in vitro* and *in vivo*, indicating the potential of pharmacologically targeting ERCC1/XPF to enhance platinum efficacy (19, 20).

First identified as a potential biomarker for response to platinum-based chemotherapy in the late 1990s, low ERCC1 expression was observed in a relatively high amount of patient tumors including in non-small cell lung, head and neck cancers and ovarian serous adenocarcinomas. While preclinical data was promising, many challenges faced the clinical implementation of ERCC1 as the first platinum biomarker including problems with antibody specificity, splice variant expression, and conflicting results from clinical and preclinical studies. However, it is possible that an incomplete understanding of basic biological factors controlling sensitivity to ICL-inducing agents in the absence of ERCC1 may also have contributed to the failure of the ERCC1 clinical trials. What has become clear over the past 10 years is that a DNA-repair deficiency does not necessarily predispose to sensitivity to a

particular drug. This is most notably observed with BRCA1/2 deficiencies in the context of PARP inhibition where loss of subsequent secondary factors is capable of making BRCA1/2 tumors resistant to PARP inhibition. In the context of ICL-repair, recent evidence has shown that loss of p53, the deubiquitinase, USP48, or the Blm-RMI1-TOPBIIa signaling axis is capable of increasing resistance of Fanconi Anemia deletion mutants to ICLs both *in vitro* and *in vivo* (21–23). In particular these findings directly implicate increased reliance on DNA repair pathways to deal with interstrand crosslinks that would otherwise be unrepaired as a result of loss of canonical ICL-R.

In this study, we identified p53 status as at least a partial modifier of the sensitivity of ERCC1[−] cells to ICL-inducing agents. Here, we characterize a panel of ERCC1[−] lung cancer cell lines developed with CRISPR-Cas9. We describe a differential phenotype in sensitivity to cisplatin and mitomycin C (MMC) that appears to be correlated with p53 status where ERCC1[−]/p53^{WT} cell lines exhibit hypersensitivity to ICL-inducing agents, but ERCC1[−]/p53^{mutant/null} cells exhibit only mild sensitivity. Finally, we show evidence that tolerance to interstrand crosslinks with ERCC1 deficiency is supported by entry into S-phase and relies on BRCA1 and DNA-PKcs function. Together this evidence suggests that functional loss of p53 may allow for the uncovering of alternate repair mechanisms capable of at least partially overcoming the repair defects associated with loss of ERCC1/XPF activity thus leading to the identification of a new subset of cisplatin-tolerant ERCC1-deficient tumors. These findings have direct clinical ramifications for future studies of ICL-repair in human tumors as well as impacting any attempts to implement biomarkers for sensitivity to ICL-inducing agents in the future.

MATERIALS AND METHODS

Cell Lines and Cell Culture:

H1299, H460, H522, H1703, H1650, H358 were all obtained from the American Type Culture Collection (Manassas, VA), were tested for mycoplasma and authenticated by the Biobanking and Correlative Sciences Core Facility at Karmanos Cancer Institute. A549 WT and ERCC1[−] cells were obtained from Jean-Charles Soria, Ken Olaussen, and Luc Friboulet (Gustave Roussy Cancer Center). OV2008 and C13* cells were obtained from Stephen B. Howell (University of California San Diego). A549 and OV2008 cells were not further authenticated or tested for mycoplasma. Cell lines were maintained for no greater than ~15 passages during the course of experiments. H1703, H522, H460, OV2008, C13, H1650, H358, and H1299 cells were cultured in RPMI-1640 (Dharmacon) media supplemented with 10% FBS (Atlanta Biologicals) and 1% Penicillin/Streptomycin (Dharmacon) and grown at 37°C in 5% CO₂. A549 cells were cultured in DMEM (Dharmacon) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% MEM, non-essential amino acids (Dharmacon), and 1% HEPES (Dharmacon).

CRISPR-Cas9 Mediated Gene Knockout:

Knockout experiments were performed essentially as previously described (Addgene plasmid 52961) (20). Knockout clones were validated by Sanger sequencing unless otherwise stated. crRNA sequences are included in Supplemental Table S1.

Colony Survival Assays:

Colony survival assays were performed as previously described (9). Cells were treated with cisplatin (Sigma-Aldrich) or MMC (Selleckchem) for 2 hours or gemcitabine (Selleckchem), camptothecin (Selleckchem), or etoposide (Selleckchem) for 4 hours in serum-free medium. Cells were treated with Palbociclib (Selleckchem), Ribociclib (Selleckchem), DNA-PK inhibitor (NU-7441; Selleckchem), or XL-413 (DBF4-dependent kinase inhibitor; Tocris) for 24 hours in complete medium. For UV-C treatment, 2,000 cells were seeded in 6-well plates and treated with the corresponding UV-C dose the following day. Plates were fixed and stained with crystal violet 3 days post-treatment and crystal violet was dissolved in 10% acetic acid and absorbance (595 nm) was measured using a SpectraMax M5 plate reader (Molecular Devices). IC₅₀s were estimated using SigmaPlot 10.0 Software.

Viability Assays:

12,000 cells were seeded in 24-well plates. Cells were treated with cisplatin for 24 hours and allowed to grow for an additional 24 hours. Live/dead cells were counted using Trypan Blue exclusion and 100 cells were counted for each concentration.

Flow Cytometry:

Apoptosis was measured by flow cytometry using the PE Annexin V Apoptosis Detection Kit (BD Biosciences). Cell cycle profiles were determined using the Propidium Iodide (PI) Flow Cytometry Kit (Abcam). For both assays, 5×10^5 cells were seeded in 10 cm plates. The following day cells were treated with 500 nM cisplatin for 2 hours in serum-free medium and cells were allowed to grow for 48 hours (for apoptosis) or were collected at various time points (cell cycle). Flow cytometry was performed on a BD LSR II SORP Flow Cytometer (BD Biosciences). Data were analyzed using ModFit LT (Verity Software House) and FlowJo v10 (FlowJo, LLC).

Modified Alkaline Comet Assay:

Modified alkaline comet assays were performed essentially as previously described (9, 20, 24). Cells were seeded in 6-well plates so that they would be ~70–90% confluent at the time of harvesting. H522 and H1299 cells were treated with cisplatin for 2 hours. Control and cisplatin-treated cells were then treated with 100 μ M H₂O₂ (Fisher Scientific) for 15 minutes immediately prior to harvesting by trypsinization at 0-hrs, 24-hrs, and 48-hrs post-cisplatin treatment. Cells were embedded in 0.5% low-melting agarose (Fisher Scientific, Catalog #BP165–25) and spread on slides coated with 1.5% Standard Low –mr Agarose (BioRad, Catalog #162–0100) and allowed to solidify. After 10 minutes, slides were placed in 4° C lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Triton X-100, pH 10) for ~1 hr. Excess buffer was removed and slides were placed in the electrophoresis tank with 4° C alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) and incubated for 20 minutes. Slides were electrophoresed for 25 minutes at 300 mA (~22–26 V). Slides were then incubated with 4° C neutralization buffer (0.4 M Tris Base, pH 7.5) for 10 minutes. Slides were fixed in 95% ethanol for 10 minutes and allowed to dry followed by incubation with SYBR-Gold (Invitrogen). Slides were imaged with a Nikon epifluorescence microscope. ~50 cells were analyzed per slide with Comet Assay Software 5.5F (Kinetic Imaging,

Liverpool, UK). ICLs were measured as the ratio of the median tail moment of the treated compared to the untreated sample where the ratio at 0-hrs post-cisplatin treatment was normalized to 100% for each isogenic cell line.

Patient Survival Analysis:

The Cancer Genome Atlas (TCGA) provisional lung adenocarcinoma cohort was utilized to assess the relationship of ERCC1 tumor expression and *TP53* mutational status on patient outcomes (25, 26). Tumor genomic and patient outcomes data were accessed for these TCGA patients on cBioPortal (26, 27). Genomic data was cleaned and normalized prior to release as described previously (25, 26). ERCC1 expression was stratified into two groups, high and low, at the upper quartile of expression values. *TP53* mutation status was also stratified into two categories, mutated or wildtype, based upon the presence or absence of nonsynonymous mutations in the coding region of the gene as detected by whole-exome sequencing. Patient overall-survival was modeled in R (version 3.4.3) using the Kaplan-Meier method and log-rank test. Because treatment data for the TCGA lung adenocarcinoma cohort is not publically available, we also analyzed the 2017 TCGA ovarian cancer data set. Patients with stage 3+4 disease who received a platinum agent were included in the analysis. Patients were selected based upon the presence or absence of a *TP53* mutation and patients were stratified based upon ERCC1 expression using the Affymetrix probe ID: 203720_s_at and the “auto select best cutoff” function. Data were analyzed using KMPlotter (kmplotter.org/ovar/) (28).

Immunofluorescence:

Cells were seeded on coverslips and treated with 500 nM cisplatin for 2 hours. 48 hours post-treatment cells were fixed with 4% paraformaldehyde and permeabilized in 0.3% Triton-X in PBS. Cells were blocked in 10% FBS in 0.1% Triton-X in PBS for 1 hr and incubated with primary antibody for 1hr and secondary antibody for 1.5 hrs in 1% BSA/ 0.1% Triton-X. DNA was stained with 300 nM DAPI for 5 minutes and coverslips mounted on slides with DakoCytomation Fluorescent Mounting Medium (Agilent) and sealed with nail polish. Slides were imaged with a Nikon epifluorescence microscope and images were analyzed using ImageJ software and the Find Maxima function. A minimum of 100 cells per group per experiment were analyzed. Antibodies are available in Supplemental Table S2.

shRNA knockdowns and re-expression of p53 and ERCC1:

BRCA1 and BRCA2 shRNA was purchased from Sigma-Aldrich. Sequences and identification numbers included in Supplemental Table S1. ERCC1–202 cDNA was purchased from Genscript and was cloned into pCDH-puro lentiviral vector. shRNA lentivirus and ERCC1–202 lentivirus was produced and transductions were performed as previously described (29). TP53 cDNA was purchased from Origene (Catalog# R200003). Cells were transfected with 2.5 µg/DNA per well with a final concentration of 3 µg/well Lipofectamine for 6 hours. Cells were allowed to rest for 24 hours, after which geneticin sulfate was added. Approximately 2 weeks post-transfection cells were harvested to assess p53 expression and experiments were performed. Knockdown of BRCA1 and BRCA2 were validated by qRT-PCR as previously described using the primers listed in Supplemental Table S1 (24).

Statistical Analysis for Cell Line Studies:

Flow cytometry and modified alkaline comet assay data were analyzed by two-sample t-test. Data comparing the dose effects of cisplatin or mitomycin c on p53 status stratified by ERCC1 WT and knockout was analyzed by two-way ANOVA with interaction test. γ H2AX foci data were analyzed by Wilcoxon rank sum test. Drug response, apoptosis, and modified alkaline comet assay experiments were all performed at least 3 times, unless otherwise stated. Cell cycle and γ H2AX foci formation experiments are presented as a representative result from 2–3 individual experiments.

Western Blots:

Protein extraction and western blots were performed as previously described (24). Antibodies used for Western Blots are available in Supplemental Table S2.

RESULTS

ERCC1 cells exhibit two distinct phenotypes upon cisplatin treatment

We developed a panel of ERCC1 cell lines using CRISPR-Cas9 to assess differences in sensitivity to ICL-inducing drugs (Figure 1A). ERCC1 has four known splice variants which differ by single intron inclusion or single exon exclusion (30), so in order to generate a clean background for our studies, we designed a crRNA targeting ERCC1 exon 2, which is shared by all ERCC1 splice variants. We utilized lung cancer cell lines that differed in p53, EGFR, and K-Ras status (Supplemental Figure S2A). As expected, loss of ERCC1 led to loss of XPF expression, since both factors generally require each other for stability. However, a self-dimerization based mechanism for XPF stability in the absence of ERCC1 has been reported in biochemical studies and could explain why one H1299 ERCC1 clone did not have reduced XPF expression (31) (Supplemental Figure S2B). Additionally, we received A549 ERCC1 cells for our investigations (Fig 1A).

Since loss of ERCC1 is necessary for key aspects of NER, HR, and ICL-R, we expected that ERCC1 loss would hypersensitize cells to cisplatin and MMC. Interestingly, upon titration of cisplatin in clonogenic and viability assays, we saw two distinct phenotypes, hypersensitivity and modest tolerance. H522, A549, and H460 ERCC1 (p53^{WT}) cells were all very sensitive to cisplatin in both clonogenic (IC₅₀s ranging from ~60 – 240 nM) and viability assays (Supplemental Figure S1A & C). These observations were validated in a second ERCC1 clone in H460 cells which showed the same hypersensitive phenotype (Supplemental Figure S2B and C). Interestingly, loss of ERCC1 in H1650, H1703, H358, and H1299 cells (p53^{null/mutant}) only resulted in modest increased sensitivity to cisplatin in clonogenic (IC₅₀s ranging from ~1.1 – 2.0 μ M) and viability assays (Supplemental Figure S1B & D). These effects were validated with multiple ERCC1 clones developed with two crRNAs, and a XPF clone in H1299 cells, suggesting this modest sensitivity is a true phenotype of loss of functional ERCC1-XPF (Supplemental Figure S2B, D-F). We were able to fully restore resistance to cisplatin in H1299 ERCC1 cells and partially restore cisplatin resistance in H460 ERCC1 cells when ERCC1–202 was re-expressed (Supplemental Figure S2G & H). We also observed this differential phenotype with MMC, a more potent inducer of interstrand crosslinks than cisplatin (Supplemental Figure S3A & B).

Conversely, we did not observe increased sensitivity of ERCC1 compared to ERCC1 WT cells with etoposide, gemcitabine or camptothecin in clonogenic assays, but both H460 and H1299 ERCC1 cells were sensitive to UV-C irradiation (Supplemental Figure S3C & D; S4A & B).

We observed that this differential phenotype appeared to be correlated with p53 status and so we performed clustering analysis based upon ERCC1 and p53 status. No differential clustering was observed in ERCC1 WT cells stratified by p53 status in the clonogenic survival or viability assays after cisplatin or MMC treatment (Figure 1B, D and F). However, plotting all ERCC1 cell lines together displayed two distinct phenotypes that appeared to be correlated with p53 status, where p53^{WT}/ERCC1 cells were significantly more sensitive to cisplatin and MMC compared to p53^{mutant/null}/ERCC1 cells (Figure 1C, E and G).

Altering p53 status alters the differential sensitivity of ERCC1 cells to cisplatin

The differential phenotype of ERCC1 cells to cisplatin and MMC appeared to be correlated with p53 status, and we hypothesized that altering p53 status could alter or reverse the observed phenotypes. To test this hypothesis, we overexpressed WT p53 in the p53-null H1299 cell line and assessed clonogenic potential after cisplatin treatment (Figure 2A & Supplemental Figure S5A). We observed that expression of p53 in parental and ERCC1 cells increased sensitivity to cisplatin (WT: 4.4 vs. 2.3 μ M and ERCC1 : 1.3 vs. 0.4 μ M) (Figure 2A). In order to address whether loss of p53 could increase resistance of p53^{WT}/ERCC1 cells to cisplatin, we utilized CRISPR-Cas9 to disrupt the *TP53* gene in H460 and H522 parental and ERCC1 cells using a crRNA targeted to Exon 7 of *TP53*. Western blots show loss of p53 in the H460 cell lines at steady state levels and upon induction with Nutlin-3 (Supplemental Figure S5A). Additionally, we confirmed disruption of *TP53* by DNA sequencing (Supplemental Figure S5E). In H460 ERCC1 /p53* cells, we observed modest increased clonogenicity (~3-fold) after platinum treatment compared to ERCC1 alone in shorter duration colony assays (Figure 2A). This fold difference could be dramatically enhanced (~10-fold) by extending the length of the colony assay from 6 to 12 days (Supplemental Figure S5B). Despite reports that H522 cells harbor a homozygous single-base deletion in codon 191 of the *TP53* gene, we were not able to detect this deletion when sequencing exons 5 and 6 of *TP53*. Therefore, for the purposes of this study, we considered the H522 cell line p53 WT. The p53-edited H522 cell lines were validated by sequencing which showed that the *TP53* alleles were disrupted by CRISPR-Cas9 in the H522 p53* cells, including an 8 amino acid in-frame deletion in one allele which would account for a slightly reduced molecular weight band near 50 kDa; we also observed the acquisition of a truncated p53 mutant near 25 kDa (Supplemental Figure S5A & E). In H522 ERCC1 cells *TP53* was partially disrupted (Supplemental Figure S5E). This would be consistent with western blot results showing induction of p53 in the ERCC1 /p53* clone (Supplemental Figure S5A). Partial p53 disruption in H522 cells also resulted in increased colony formation after cisplatin treatment in the ERCC1 cells (Figure 2A). Similar results were also observed in A549 WT and ERCC1 cells upon *TP53* disruption (Supplemental Figure S5C-E).

We also assessed changes in viability of H460 and H522 isogenic cell lines with increasing doses of cisplatin by Trypan Blue live/dead assays. Strikingly, we saw that disruption of p53 in ERCC1^{-/-} H460 cells increased the IC₅₀ in viability assays ~15-fold (100 nM vs. 1.5 μ M) (Figure 2B). In H522 cells, partial disruption of *TP53* in ERCC1^{-/-} cells increased the IC₅₀ in viability assays ~2-fold (1.5 μ M vs. 3 μ M) (Figure 2B). We also performed flow cytometry-based analysis of apoptosis with H460 and H522 isogenic cell lines. All paired cell lines were treated for 24 hours with the IC₅₀ dose of the ERCC1^{-/-} determined in the Live/Dead Assays and cells were allowed to grow for an additional 24 hours before proceeding with flow cytometry. In H460 and H522 ERCC1^{-/-} cells, cisplatin treatment resulted in approximately 50% cell death as measured by the percent Annexin-V positive cells (Figure 2C). In ERCC1^{-/-}/p53* cells, loss of p53 conferred significant protection from cisplatin-induced apoptosis (Figure 2C). Additionally, the level of apoptosis observed in ERCC1^{-/-}/p53* cells was not statistically different from p53* cells. These viability data appear to suggest that p53 loss is critical for limiting apoptosis in the presence of unrepaired ICLs with loss of ERCC1.

Next, we tested whether knockout of ERCC1 had differential effects on cisplatin sensitivity in the OV2008/C13* cell line model of cisplatin resistance. C13* cells exhibit increased levels of ERCC1 compared to OV2008 cells (Supplementary Figure S5F). While both cell lines possess wild-type p53, p53 induction in C13* cells is impaired and p53 is not stabilized upon platinum treatment (Supplemental Figure S5G) (32, 33). In clonogenic assays, we observe OV2008 ERCC1^{-/-} cells are more sensitive to cisplatin than the C13* ERCC1^{-/-} cells (Supplementary Figure S5H). This suggests that the differential phenotype may not be limited to p53 mutations but could be extended to include defects in p53 stability/induction.

Kinetics of the DNA damage response in ERCC1^{-/-} cells

Our hypothesis regarding the role of p53 in this differential phenotype was that p53 predisposed repair-deficient cells to apoptosis and that loss of p53 promoted DNA damage tolerance. In order to assess general levels of DNA damage signaling in the ERCC1^{-/-} cells, we performed a treatment time-course and measured levels of p53, CDKN1A (p21), PARP1 cleavage, and H2AX phosphorylation at various time points by western blot. In the cisplatin-hypersensitive H460 and H522 ERCC1^{-/-} cells, we saw induction of PARP cleavage after treatment, consistent with the viability assays (Figure 3A). Additionally, we saw induction of p53 and p21 over time which persisted (Figure 3A). Consistent with previously reported observations (5), we detected a large induction of γ H2AX in the H460 and H522 ERCC1^{-/-} cell lines which continued to the 48-hour time-point indicating persistent, unrepaired DNA DSBs in the hypersensitive cells (Figure 3A). H1299 ERCC1^{-/-} cells exhibited very little induction of cleaved PARP after treatment with cisplatin (Figure 3A). Unexpectedly, there was very little induction of γ H2AX in H1299 ERCC1^{-/-} cells compared to WT cells (Figure 3A), indicating either 1. DNA damage signaling is defective, or that 2. DSB repair is not defective in these cells. The former possibility is unlikely, considering cells defective in phosphorylation of H2AX are sensitized to DNA damaging agents such as ionizing radiation (34–36). This evidence suggests differential responses to DNA damage may contribute to the bimodal phenotype observed in our panel of ERCC1^{-/-} cells.

We further confirmed γ H2AX results from western blots by immunofluorescence. In H460 WT cells, cisplatin treatment did not result in increased γ H2AX foci formation 48 hours post-cisplatin treatment, while in ERCC1^{-/-} cells foci formation was dramatically increased (Figure 3B). These data would suggest that DSB formation during ICL-R is at least partially independent of ERCC1/XPF activity. This would be consistent with previous reports in ERCC1 deficient cells showing other endonucleases are capable of the initial incision steps of ICL-R including Mus81 and Fan1 (reviewed in (37)). In H460 ERCC1^{-/-}/p53⁺ cells we observed a significant reduction in the number of γ H2AX foci present 48 hours after treatment (Figure 3B). Conversely, in cisplatin-tolerant H1299 ERCC1^{-/-} cells we observed very few γ H2AX foci at 48 hours post-cisplatin treatment likely suggesting that ICL-R is largely not defective in these cells (Figure 3B).

Cisplatin is a bifunctional drug which induces ISAs between guanine residues on the same strand of DNA and these structures, which are repaired through NER, represent >90% of the damage caused by cisplatin while ICLs represent approximately 1–5% of total DNA adducts generated by cisplatin. We suspected that ISAs, which should persist in ERCC1^{-/-} cells, may be less toxic than ICLs which can function as complete replication blocks. In order to tease apart whether the modest sensitivity we observe in H1299 ERCC1^{-/-} cells in clonogenic assays is due to unrepaired ISAs, we generated XPA^{-/-} clones in H1299 cells (Supplemental Figure S6A & D). XPA's only described function is to act as a scaffolding protein during NER, and XPA-deficient cells are less sensitive to MMC than ERCC1-deficient cells (38). So XPA^{-/-} cells should display the relative contribution of unrepaired ISAs in H1299 cells. In clonogenic assays, XPA^{-/-} cells display the same sensitivity to cisplatin as ERCC1^{-/-} or XPF-deficient cells, strongly pointing to the modest sensitivity observed as the relative contribution of unrepaired ISAs (Supplemental Figure S6B & E). We also measured sensitivity to MMC which induces monoadducts and a higher level of ICLs than cisplatin. We observed a classic phenotype, where ERCC1 and XPF knockout cells were ~2.5-fold more sensitive to MMC than XPA^{-/-} cells (Supplemental Figure S6C). We hypothesize that the relative amount of ICLs compared to the total amount of DNA damage may impact this differential phenotype with ERCC1 deficiency. To assess differences in ICL-R between our isogenic cell lines, we performed modified alkaline comet assays in the H522 and H1299 isogenic cell lines. While this assay is an indirect measure of interstrand crosslinked DNA, it is commonly used to measure platinum ICL-R. H1299 cells were treated with 5 μ M and H522 cells with 1.5 μ M cisplatin for 2-hours followed by measurements of ICL-R at the 0 (immediately after treatment), 24, and 48-hr time-points. Despite attempts to perform these analyses with H460 isogenic cell lines, the ERCC1^{-/-} cells were too sensitive to cisplatin to observe significant differences between untreated and treated samples at the 0 hr time point when treated with 500 nM cisplatin, making it impossible to accurately monitor ICL DNA repair via this assay. As we expected, H522 ERCC1^{-/-} cells were not capable of ICL-R (Figure 3C). In H522 ERCC1^{-/-}/p53⁺ cells, ICL-R was at least partially rescued where there was no difference compared to WT or p53⁺ cells at the 24-hour time point, but statistically greater amounts of ICL DNA damage remained at 48 hours post-treatment relative to p53⁺ cells and less amounts of damage remained relative to ERCC1^{-/-} cells (Figure 3C). This observation would be consistent with partial disruption of *TP53* in these cells. Consistent with the DNA damage signaling we observed in H1299 ERCC1^{-/-} cells, no delay in ICL-R

compared to H1299 WT cells was detectable (Figure 3C), however, re-expression of p53 in H1299 ERCC1 cells induced a near-complete block of ICL-R compared to parental cells (Figure 3C).

Cell cycle arrest profiles differ in ERCC1 cells after cisplatin treatment

Many reports with ERCC1 knockout and knockdown cells have shown that after treatment with a crosslinking agent there is a potent G2/M cell cycle arrest which has been attributed to unrepaired DNA damage. To test whether p53 status affected cell cycle arrest after treatment with cisplatin, we assessed cell cycle profiles in a time course after treatment. While the p53 null H1299 cells exhibited no distinct G2/M arrest after platinum treatment, we consistently observed a slight increase in G2/M arrest in the H1299 ERCC1 cells at the 24-hour time-point which resolved by the 48-hour time-point (Figure 4A & C). Conversely, in H460 ERCC1 cells we observed a potent G2/M arrest 24 hours post-cisplatin treatment (Figure 4B & C). By 48 hours post-treatment, we detected a sharp increase in a sub-G1 population consistent with an increase in cell death. In the H460 ERCC1 /p53*cells we observed the same potent G2/M arrest that we observed in H460 ERCC1 cells at 24 hours post-treatment (Figure 4B & C). Astonishingly, by 72 hours after treatment we saw a near-complete recovery from G2/M arrest with only a minor increase in the sub-G1 population. We hypothesize that unrepaired DNA damage leads to G2/M arrest but that this G2/M arrest is not permanent. Eventually cells enter into M-phase and subsequently into G1 phase where the presence of DNA DSBs triggers p53-mediated cell death. However, in the absence of p53, cells are either capable of tolerating unrepaired DNA crosslink damage or alternate repair mechanisms may exist that can at least partially compensate for loss of ERCC1.

p53 status may act as a confounding variable in clinical assessments of ERCC1 as a platinum biomarker

The potential for utilizing ERCC1 expression to predict clinical response to platinum-based chemotherapy has been extensively tested in multiple cancer types including lung and ovarian cancers with varying results (39–42). We wanted to assess whether p53 status may be a confounding variable. Utilizing the TCGA lung adenocarcinoma data set, we split patients into two groups; those whose tumors had WT p53 and those whose tumors had any amino acid-changing mutation in p53. While we did not observe any significant difference in ERCC1 expression between groups ($p=0.156$), when we stratified wild-type p53 tumors based upon ERCC1 high or low expression, we observed a significant ~50% increase in median overall survival (OS) for patients with low ERCC1 (Figure 5A & B, D). However, in patients whose tumors had p53 mutations, no significant increase in median OS for patients with low compared to those with high ERCC1 was observed (Figure 5C & D). While TCGA lung adenocarcinoma treatment data is not publically available, nearly 100% of lung adenocarcinoma patients receive a platinum agent during the course of treatment, and so we hypothesize that these data are contingent upon platinum treatment. We also corroborated these results in the TCGA ovarian cancer data set in terms of both progression-free and OS specifically in the context of platinum treatment (Supplemental Figure S7). We did not specifically test the effects of including BRCA1/2 mutation status in our analysis, however, BRCA1/2 mutations are generally observed in a p53 mutant context where we did not observe a significant benefit for patients with low ERCC1 in the context of OS.

Mechanistic characterization of ICL tolerance with ERCC1-deficiency

We hypothesized that in a p53^{mutant/null} background where apoptosis and G1 checkpoint activation are diminished, alternate repair mechanisms may exist to deal with the damage from unrepaired ICLs that accumulate as result of loss of ERCC1. It is well documented that ERCC1/XPF activity is critical for ICL-R in G1 phase, where replication-dependent processes are not available for dealing with ICLs, so we tested whether transient inhibition of entry into S phase could sensitize ICL-tolerant ERCC1^{-/-} cells to cisplatin. Palbociclib alone inhibited growth of H1299 WT and ERCC1^{-/-} cells and corresponded with an increase in the percentage of cells in G1 phase compared to untreated cells (Supplemental Figure S8C & D). For combination treatment experiments, we treated cells with cisplatin followed by 24-hour treatment with the CDK4/6 inhibitors, palbociclib and ribociclib. We observed that H1299 WT and ERCC1^{-/-} cells could be sensitized to cisplatin in clonogenic assays even with transient inhibition of CDK4/6 activity (Figure 6A & Supplemental Figure S8B) which was very similar to what we observed with reexpression of p53 (Figure 2A). The addition of palbociclib to hypersensitive H460 or H522 ERCC1^{-/-} cells did not further enhance cisplatin sensitivity, although there were small increases in sensitivity for the parental cell lines (Supplemental Figure S8A & B). Furthermore, blocking replication initiation in H1299 ERCC1^{-/-} cells via inhibiting the DBF4-dependent kinase (DDK) with the inhibitor, XL413, could also sensitize to cisplatin (Figure 6A). We take this to suggest that in hypersensitive ERCC1^{-/-} cells, ICL-R is completely dependent upon ERCC1/XPF activity whether or not cells are in G1 or S-G2-M phases of the cell cycle. On the other hand, the increased sensitivity of H1299, H460, and H522 WT cells with CDK4/6 or DDK inhibition may indicate that timely entry into S-phase is also critical for supporting platinum resistance despite being DNA repair proficient. This requirement for S-phase entry appears to be exacerbated in p53 null H1299 ERCC1^{-/-} cells where platinum sensitization by CDK4/6 or DDK inhibition indicates that ERCC1/XPF activity is critical for ICL-R in G1 phase in ICL-tolerant cells, and that entry into S-phase may lead to ERCC1/XPF-independent mechanisms for tolerating or repairing ICL-DNA damage.

Next, we tested whether factors involved in non-homologous end joining (NHEJ) and HR supported the tolerance observed in H1299 ERCC1^{-/-} cells. Inhibition of DNA-PKcs activity with the inhibitor, NU7441, selectively increased sensitivity of ERCC1^{-/-} H1299 cells (1.3 vs. 0.5 μ M), but not parental cells, to cisplatin (Figure 6B). Additionally, we performed shRNA knockdowns of BRCA1 in H1299 cells and assessed clonogenicity after cisplatin treatment. BRCA1 knockdown led to increased sensitivity in both H1299 WT and ERCC1^{-/-} cells, consistent with critical roles for BRCA1 in regulating DSB end-resection and contributing to HR, translesion synthesis, and microhomology-mediated end joining (Figure 6C). Interestingly, the effects of BRCA1 knockdown in sensitizing ICL-tolerant ERCC1^{-/-} cells to cisplatin appear to be independent of BRCA2 as we only observed increased sensitivity in WT cells, but no increased sensitivity of ERCC1^{-/-} cells to cisplatin (Supplemental Figure S8E). Furthermore, BRCA1 knockdown led to a significant increase in the presence of γ H2AX foci persisting 48 hours post-cisplatin treatment in the H1299 ERCC1^{-/-} cells treated with cisplatin compared to ERCC1^{-/-} alone (Figure 6D). While the mechanism underlying ICL-tolerance in a subset of ERCC1^{-/-} cells is not entirely parsed out,

it appears that this tolerance is dependent upon DNA-PKcs and BRCA1 function, but likely independent of BRCA2.

DISCUSSION

A recent international randomized Phase III clinical trial utilizing ERCC1 expression to predict response to platinum-based chemotherapy did not show clinical benefit for non-small cell lung cancer patients with low ERCC1 who received a platinum agent (40, 41). Additionally, a preclinical study failed to show any correlation between pretreatment ERCC1 expression in ovarian cancers and response to platinum-based chemotherapy (42). Here we showed that our *in vitro* data may have direct clinical implications where we observed a clinical benefit for lung adenocarcinoma and ovarian carcinoma patients with low ERCC1 only when WT p53 was retained. These data may provide an additional explanation for conflicting results from clinical studies as to the benefit of using ERCC1 expression to predict responders to platinum-based chemotherapy. Our data may have the greatest impact in cancer types where the p53 mutation rate is markedly high, such as lung adenocarcinoma (~50% p53 mutant) and ovarian serous carcinoma (~90%) where ERCC1 has been investigated as a platinum biomarker.

In this study, we characterized a panel of ERCC1⁻ lung cancer cell lines. We identified a differentially sensitive phenotype of ERCC1⁻ lung cancer cell lines that appears to be at least partially associated with p53 status. If cells harbored WT p53, the ERCC1 deletion clones exhibited hypersensitivity to crosslinking agents, while the p53^{mutant/null} cell lines exhibited mild sensitivity. Viability after cisplatin treatment of ERCC1⁻ cells was dramatically increased by disrupting p53 by CRISPR-Cas9. On the other hand, clonogenicity of ERCC1⁻ cells increased by disrupting p53 while sensitivity was increased in ERCC1⁻/p53^{null} cells following expression of *TP53* cDNA. The modest increases in clonogenicity in isogenic ERCC1⁻ cells with subsequent disruption of p53 compared to our panel of ERCC1⁻ cells likely suggests additional factors, such as those involved in processing and stability of replication forks (RPA availability for example), may be critical for further enhancing clonogenicity in response to platinum with loss of ERCC1 (43). This would be most significant in terms of factors involved in response to intrastrand DNA damage which also theoretically requires ERCC1/XPF activity for resolution. A similar phenotype was observed by Feng and Jasin, where they observed a similar partial, but significant, rescue of clonogenicity of BRCA2-deficient cells with p53 loss, potentially suggesting that alterations in additional factors or pathways are critical for supporting clonogenic growth in tumors harboring a p53 mutation and loss of BRCA2 (44).

Interestingly, modified alkaline comet assays in H522 and H1299 cell lines showed differential repair of ICLs depending on p53 status. We also demonstrated that ERCC1 cells exhibit G2/M arrest following cisplatin treatment. p53 disruption in H460 ERCC1 cells did not alter the initial G2/M arrest observed in H460 ERCC1⁻ cells, however, at 48 hours post-treatment there was a dramatic increase in a sub-G1 population in H460 ERCC1 cells that corresponded with a decrease in the G2/M population. This is opposed to the near complete abrogation of cell death and G2/M arrest in the H460 ERCC1⁻/p53^{*} cells.

Of importance, transient inhibition of entry into S-phase sensitized ICL-tolerant ERCC1 / p53* cells to cisplatin, suggesting ERCC1/XPF is indeed critical for ICL-R in G1 phase and that the persistence of these unrepaired ICLs may trigger growth inhibition. This growth inhibition also suggests that entry into S-phase is critical for supporting ICL tolerance in these cells where there may be decreased dependence on ERCC1/XPF for ICL unhooking resulting in the accumulation of DNA DSBs. Based upon our data, it is likely that loss of downstream functions of ERCC1/XPF in ICL-R lead to persistent DSBs that are unresolved, at least initially, leading to G2/M arrest. However, cells eventually escape this arrest and enter into M and subsequently into G1 phase where p53 activity is critical for sensing persistent DNA damage from the previous round of the cell cycle and triggering apoptosis as well as activating the G1 checkpoint. It is most likely that loss of p53 leads to a decrease in this apoptotic potential and loss of G1 checkpoint activation which enables secondary, alternate repair pathways to at least partially contribute to ICL-R either in later stages of the cell cycle where a number of error-prone repair pathways, independent of BRCA2, may be available, including break-induced replication, microhomology-mediated end joining, and NHEJ. This hypothesis is supported by the observation that DNA-PKcs and BRCA1, but not BRCA2, are critical for supporting tolerance to ICLs in the absence of p53 and ERCC1. A similar phenotype with BRCA1 was recently reported in MMC-resistant, FANCC-deficient cells where BRCA1 function was critical for supporting MMC resistance in the absence of USP48, despite loss of canonical ICL-R (22). Together these data suggest that in both p53 and ICL-R deficient cells there is the potential uncovering of alternate DNA repair or tolerance mechanisms for dealing with unrepaired ICLs that specifically relies upon DNA-PKcs and BRCA1 as well as entry into S phase.

Several other groups have identified similar differential phenotypes *in vitro* and *in vivo* with loss of factors involved in ICL-R that appear to be correlated with p53 status including Mus81, BRCA2, and FANCD2 (21, 44, 45), although a mechanism for this differential phenotype has not been described. However, the role of p53 in inducing apoptosis does not appear to fully account for this differential phenotype as a recent report showed in FANCD2-deficient mice that p53 loss completely rescued mice from Fanconi Anemia symptoms specifically in the context of aldehyde-DNA ICLs (21). While the abrogation of FA symptoms was certainly related to a reduction in apoptosis, the authors also observed a dramatic increase in chromosomal aberrations including deletions and translocations suggesting that loss of p53 may uncover an alternate, error-prone ICL-R pathway and that WT p53 serves to suppress this error-prone repair likely through its roles in controlling apoptosis and mediating cell cycle control.

In conclusion, the work in this study characterizes a novel phenotype of ICL-tolerance in a subset of ERCC1-deficient cells and highlights the potential importance of p53 as a clinically relevant variable in studies evaluating ERCC1, and possibly other ICL-R factors, as a platinum biomarker. The surprising finding that DNA-PKcs and BRCA1 support this phenotype of resistance suggests there are repair mechanisms in place which can at least partially overcome the ICL-R defects associated with loss of ERCC1. Furthermore, it will be important to fully characterize the molecular mechanisms underlying this process and to expand the list of repair factors that are involved in supporting resistance to crosslinking agents despite loss of canonical ICL-R.

Better understanding mechanisms of resistance to DNA crosslinking agents in the context of DNA repair deficiencies may lead to the identification of novel targets for therapeutic intervention that could be developed to improve patient responses to platinum-based chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

There remains a longstanding interest in understanding how to use tumor DNA-repair deficiencies to predict response to chemotherapy and immunotherapy. Perhaps the best example of such an endeavor was the testing of ERCC1 expression as a platinum biomarker in non-small cell lung cancers in an international, randomized Phase III trial which ultimately did not show clinical benefit for patients with low ERCC1 receiving platinum. This was surprising considering ERCC1/XPF activity is thought to be essential for repair of platinum-DNA damage. We have identified a synthetic viable phenotype of tolerance to cisplatin in a panel of ERCC1-deficient cells that appears to be partially controlled by p53, thus leading to the identification of a new subset of platinum-tolerant, ERCC1-deficient tumors. Furthermore, this differential response to platinum was also observed in two patient cohorts, suggesting p53 status as a potential confounding variable in clinical studies attempting to implement ERCC1 as a platinum biomarker.

Figure 1. Cisplatin and mitomycin C sensitivity of a panel of ERCC1 lung cancer cell lines. **A.** Western blot depicting ERCC1 and XPF expression in the WT and ERCC1 cell lines generated by CRISPR-Cas9 and the A549 WT and ERCC1 cells. **B-C.** Clustering of cisplatin clonogenicity assays of ERCC1 WT and ERCC1 cells by p53 status. **D-E.** Clustering of cisplatin viability assays of ERCC1 WT and ERCC1 cells by p53 status. **F-G.** Clustering of MMC clonogenicity assays of ERCC1 WT and ERCC1 cells by p53 status. Data analyzed by two-way ANOVA. *** $p < 0.001$

Figure 2. Effects of p53 on sensitivity of ERCC1 cells to cisplatin.

A. Clonogenic survival after cisplatin treatment in H1299 (p53-null and p53 re-expressed), H460 (p53 WT and knockout), and H522 (p53 WT and knockout) isogenic cell lines differing by p53 and ERCC1 status. $n=3$; data plotted as average of three independent experiments $\pm$ SD. **B.** Cisplatin viability assays of H460 and H522 isogenic cell lines treated with escalating doses of cisplatin., $n=3$; data plotted as average $\pm$ SD. **C.** Compilation of data for H460 and H522 cells from three independent flow cytometry experiments representing % Annexin-V positive cells $\pm$ single-dose cisplatin treatment. Data represented

as average % Annexin-V positive cells $\pm$ SD. * $p < 0.05$ measured by two-sided t-test. NS = No Significance, $p > 0.05$.

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Figure 3. Differential DNA damage signaling and interstrand crosslink repair in ERCC1 cells.
A. Western blots of a cisplatin treatment time course measuring induction of cleaved PARP, γ H2AX, p53 and p21 up to 48 hours in H460, H522, and H1299 WT and ERCC1 cells. **B.** Images and Quantification of one representative experiment showing γ H2AX foci formation in H1299 and H460 cells 48 hours post-cisplatin treatment. **** $p < 0.0001$ as measured by Wilcoxon rank sum test; experiments performed 3 times. **C.** Modified alkaline comet assay data indirectly measuring ICL-R in H522 and H1299 cells. Data presented as mean of three independent experiments. Error bars represent SEM. $p < 0.05$ as measured by student's t-test.

Figure 4. Cell cycle profiles after cisplatin treatment in ERCC1 isogenic cell lines. Representative data from flow cytometry experiments measuring cell cycle profiles in **A.** H1299 and **B.** H460 isogenic cells at varying time points after cisplatin treatment. (n=2 for each sample). **C.** Quantification of the percent of cells in G2/M phase following cisplatin treatment. Analysis excludes the sub-G1 population of cells from the quantification.

Figure 5. ERCC1 expression and p53 status in relation to OS in the TCGA lung adenocarcinoma data set.

A. Expression of ERCC1 in lung adenocarcinoma tumors delineated by p53 status. Data compared by student's t-test. OS of lung adenocarcinoma patients whose tumor harbored **B.** WT p53 and stratified by ERCC1 expression, or **C.** mutated p53 and stratified by ERCC1 expression. **D.** Combined model of OS of lung adenocarcinoma patients accounting for p53 status and ERCC1 expression.

Figure 6. Molecular pathways contributing to cisplatin tolerance in p53-null cells with ERCC1 deficiency.

A. Clonogenic survival assays of H1299 WT and ERCC1 Δ cells treated with cisplatin and palbociclib, ribociclib, or DBF4-dependent kinase inhibitor. Left panel: Plot depicts one representative experiment (n=3). Middle panel: Plot represents average of three independent experiments. Right panel: Plot represents one representative experiment (n=3). **B.** Clonogenic survival of H1299 isogenic cells treated with cisplatin $\pm$ NU7441 (DNA-PKcs inhibitor) (n=3). **C.** Western blot showing BRCA1 knockdown and clonogenic assays of H1299 WT and ERCC1 Δ with shControl and shBRCA1 knockdown. **D.** Quantification of γ H2AX foci formation 48 hours post-cisplatin treatment in H1299 WT and ERCC1 Δ BRCA1 knockdown cells $\pm$ cisplatin treatment. Data is representative of 2 independent

experiments. **** $p < 0.0001$ as measured by Wilcoxon rank sum test. **E.** Model depicting proposed mechanistic hypothesis regarding ICL-tolerance in ERCC1/p53-deficient cells.

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