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Targeting Germline and Tumor Associated Nucleotide Excision Repair Defects in Cancer

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Abstract

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Conflict of interest statement: JV, ST and KO are inventors on a patent application for use of Illudin class of alkylating agents in patients harboring mutations in the ERCC3 gene (PCT/US2018/022588). MWM has a financial interest in irofulven. JER reported receiving consulting fees/trial funding from Bayer, Seattle Genetics, AstraZeneca, Roche/Genentech, Astellas and Mirati. Trial funding from Novartis and Jounce, consulting fees from BMS, Merck, Western oncology, Adicet Bio, Sensei Biotherapeutics, QED Therapeutics, Bioclin Therapeutics, Pharmacyclics, Fortress Biotech and Janssen as well as a Honorarium from Chugai.

Purpose: Nucleotide excision repair (NER) gene alterations constitute potential cancer therapeutic targets. We explore the prevalence of NER gene alterations across cancers and putative therapeutic strategies targeting these vulnerabilities.

Experimental Design: We interrogated our institutional dataset with mutational data from more than 40,000 patients with cancer to assess the frequency of putative deleterious alterations in four key NER genes. Gene-edited isogenic pairs of wildtype and mutant *ERCC2* or *ERCC3* cell lines were created and used to assess response to several candidate drugs.

Results: We found that putative damaging germline and somatic alterations in NER genes are present with frequencies up to 10% across multiple cancer types. Both *in vitro* and *in vivo* studies showed significantly enhanced sensitivity to the sesquiterpene irofulven in cells harboring specific clinically observed heterozygous mutations in *ERCC2* or *ERCC3*. Sensitivity of NER mutants to irofulven was greater than to a current standard of care agent, cisplatin. Hypomorphic *ERCC2/3* mutant cells have impaired ability to repair irofulven induced DNA damage. Transcriptomic profiling of tumor tissues suggested co-dependencies between DNA repair pathways, indicating a potential benefit of combination therapies, which were confirmed by *in vitro* studies.

Conclusions: These findings provide novel insights into a synthetic lethal relationship between clinically observed NER gene deficiencies and sensitivity to irofulven and its potential synergistic combination with other drugs.

Keywords

DNA repair; nucleotide excision repair; biomarker discovery; cancer genomics; synthetic lethality

Germline mutations in DNA repair genes are a common cause of hereditary cancer predisposition. Defects in genes that regulate double strand break (DSB) repair/homologous recombination (HR), such as *BRCA1/2*, increase risk for several cancers (1-5), and confer sensitivity to Poly (ADP-ribose) polymerase (PARP)-inhibitors (6). Alterations in mismatch repair (MMR) genes are associated with increased cancer risk for colorectal, gastric, endometrial and other cancers (7), and the associated MSI-high phenotype is predictive of response to immune checkpoint inhibitors (8). Cancer risk associated with mutations in nucleotide excision repair (NER) genes is less well understood and currently there are no targeted therapies FDA approved specifically for patients with germline or somatic mutations in NER pathways genes.

The ATP-dependent DNA helicases *ERCC2* and *ERCC3* are part of the transcription factor IIH (TFIIH) complex, which is involved in RNA polymerase II mediated transcription and plays a crucial role in the process of nucleotide excision repair (NER). NER is divided into two sub-pathways, transcription-coupled NER (TC-NER) and global genomic NER (GG-NER) (9). Following recognition of DNA damage by either TC-NER or GG-NER sensors, repair is performed by a common mechanism involving unwinding DNA at the damage site by helicases *ERCC2* and *ERCC3*, incision by endonucleases *ERCC1/4/5* and subsequent error-free gap filling and ligation. Biallelic mutations in the genes encoding the core NER factors *ERCC1-5* give rise to inherited syndromes associated with increased cancer risk (10, 11).

Candidate gene studies have suggested common SNPs in *ERCC2* as a potential risk factor for increased melanoma and bladder cancer risk (12, 13). We recently showed that the *ERCC3* p.R109X variant confers partial loss of function and increased breast cancer predisposition (14). Based on these observations, other truncating *ERCC3* mutations (somatic or germline) are predicted to confer haploinsufficiency as well. Somatic *ERCC2* mutations are present in approximately 10% of bladder cancers and found at lower frequency in a range of other cancer types and have been demonstrated to correlate with increased cisplatin sensitivity (15). Thus, NER gene mutations can act as predictive biomarkers that indicate an increased risk to develop cancers for carriers of pathogenic variants in these genes and therefore can be helpful for early cancer detection when carriers are subjected to increased cancer screening. At the same time, they can also be used as prognostic biomarkers, informing physicians about increased sensitivity of tumors harboring variants in these genes to drugs targeting DNA repair deficiencies.

The fungal sesquiterpene illudin S exhibits toxicity against cells deficient for NER pathway components (16). We have previously shown that heterozygous mutations in *ERCC3* confer increased sensitivity to illudin S (14). Illudin S exhibits enhanced cytotoxicity in multidrug resistant and leukemia cells but was not suitable for clinical use due to toxicities observed in animal studies (17). Hence, efforts were undertaken to develop semisynthetic derivatives of illudin S, such as the acylfulvenes, with better therapeutic indices, that could potentially induce apoptosis in tumor cells at lower doses (18). The illudin S derivative irofulven has shown very promising anti-tumor activity in both cell lines and xenografts, especially in solid tumors (19). Illudin S and its derivative irofulven create DNA lesions that are specifically recognized by TC-NER but are ignored by GG-NER sensors (20). However, whether irofulven has selectivity for tumor cells with heterozygous mutations in *ERCC2/ERCC3* or other common NER or TC-NER pathway genes has not been characterized.

Here, we take a first step towards estimating the prevalence of putative functionally significant monoallelic NER pathway mutations in a large dataset of patients with cancer. We then investigate a potential treatment option targeted for a NER deficient patient population by assessing pre-clinical efficacy of irofulven in heterozygous *ERCC2* or *ERCC3* mutant cells *in vitro* and in isogenic cell line-, or patient-derived xenografts. We elucidate the increased efficacy of irofulven as a mechanistic consequence of hypomorphic NER function and delineate the deregulated cellular signaling pathways downstream conferring cytotoxicity. Building on these data, we identify combination therapies that can act synergistically. Overall, our data support irofulven based combinations as a precision therapy for tumors with NER mutations.

Materials and Methods

Tumor-normal sequencing and interrogation of germline and somatic mutational datasets

All germline and tumor sequencing performed in this study was completed as part of a research study. Paraffin-embedded tumor and blood from patients were obtained and sequenced using the MSK-IMPACT platform, a capture-based NGS assay capable of identifying mutations, copy number alterations, and select gene fusions involving 341 cancer-associated genes in the first iteration and 468 in the more recent iteration, as

described previously (21, 22). For the anonymized cases, sequence data were assigned a unique study identifier and irretrievably de-linked from personal identifiers before variant calling. Germline variants from the .bam file of the constitutional DNA with mapping and base quality scores of >20 were called using GATK Haplotypecaller2. Only variants with 50X depth of sequencing, were included in further analysis. For germline data, only heterozygous variants with <1% population frequency in the Genome Aggregation Database (gnomAD) were included in the analysis. Subsequent pathogenicity filters were applied and included the following criteria. Variants reported as benign and likely benign in ClinVar were excluded and we used CAVA (23), VEST (24) and our in-house algorithm PathoMAN (25) for variant annotation and in-silico prediction to filter for pathogenic and likely pathogenic variants. In addition, variants observed in the homozygous state were excluded, since these are less likely to confer loss of function if they are tolerated in the homozygous state within these critical genes. Variants showing a moderate or high impact in CAVA and PathoMAN as well as a VEST score of >0.6 were included in the final analysis set. Somatic variants were derived from MSKCC cBioPortal (26, 27). Variants were filtered to exclude those annotated as likely neutral through the OncoKB knowledge base (28) and only variants with a functional impact (FI) score >2.5 as determined by the MutationAssessor tool (29) were included in the analysis dataset.

Cell culture and treatments

HMLE cells harboring the heterozygous *ERCC3*R109X variant have been generated by our group using CRISPR/Cas9 as described previously (14). Similarly, the *ERCC2* missense variants E79D and Y24C were introduced into the HMLE cell line, heterozygous and homozygous clones were expanded and validated by PCR and Sanger sequencing. An isogenic pair of KU1919 human bladder carcinoma cells of wildtype and *ERCC2* compound heterozygous mutant [L485fs/T484_L485del] was kindly provided by the Solit lab at MSKCC. For cell viability assays these cell lines were treated with various compounds as described and cell viability was assessed at the indicated timepoints following treatment using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Western Blotting

Protein lysates were prepared in RIPA buffer (Pierce) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Samples were run on 4-12% gradient Bis-Tris SDS PAGE gels (Invitrogen), transferred onto PVDF membranes (Bio-Rad) and probed with antibodies against ERCC3 (ARP37963_P050; 1:2,500; Aviva Systems Biology), ERCC2 (ab54676; 1:1000; Abcam), phospho-Histone H2A.X (Ser139) (1:1000; Cell Signaling Technology), phospho-Chk1 (Ser345) (1:1000; Cell Signaling Technology), and GAPDH (V-18; 1:2000; Santa Cruz Biotechnology). HRP-conjugated secondary antibodies were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Flow cytometry

For flow cytometric analysis, 24h after seeding, cells were either left untreated or were treated with the indicated doses of irifolven for 2 hours. Treated cells were subsequently washed with PBS and supplemented with drug-free medium. The cells were harvested at

different time points after treatment and fixed with 4% PFA for 10min at room temperature (RT). Cells were briefly chilled on ice and ice-cold methanol was added to a final concentration of 90%. The cells were incubated on ice for 30min and stored at -20°C until further processing. For immunostaining the cells were washed twice with PBS/0.5% BSA and incubated with anti phospho-Histone H2A.X (Ser139) Antibody (Alexa Fluor® 488 Conjugate) (1:250; Cell Signaling Technology) for 1-2h at RT, washed again and incubated with Alexa-488 conjugated secondary antibody for 45min at RT. For each condition a minimum number of 20,000 cells were recorded and data from at least two replicate experiments was analyzed using the FlowJo software (V10.5.3). An example of the gating strategy can be found in Supplementary Fig. S1.

Xenograft experiments

Animal studies were approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee (IACUC) and Research Animal Resource Center (RARC). NSG (Jackson Laboratory) and athymic (nu/nu; Envigo Laboratories) mice were used for in vivo studies and were cared for in accordance with institutional guidelines. Patient-derived xenografts (PDXs) were generated as follows: 6-week-old NSG female mice were implanted subcutaneously with specimens freshly collected from patients at MSK Hospital under an approved IRB biospecimen protocol, as previously described (30). Tumors developed within 2 to 4 months and were expanded into additional mice by serial transplantation. The generated PDXs were subjected to high-coverage next-generation sequencing with the MSK-IMPACT assay (Supplementary Table S1). Isogenic cell line xenografts were generated by injecting 10 million hRAS-V12 driven *ERCC3* wildtype or heterozygous R109X mutant HMLE cells together with matrigel (50:50) subcutaneously in 6-week-old athymic female mice. In all instances, once tumors reached an average volume of 100 mm³, mice (8-10 mice/group) were randomized to receive either vehicle or iriofulven 3.5 mg/kg i.p. for 5 consecutive days. Cisplatin was given at a dose of 3.5 mg/kg i.p. once weekly to avoid excessive toxicities. The treatment cycle was repeated every 21 days for a total of three cycles. Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula: length x width² × 0.52. Body weight was also assessed twice weekly. Treatment was terminated when either significant toxicities were observed, tumors had completely regressed, or when mice had to be sacrificed due to large tumor burden. Upon completion of the experiments the tumors were excised, flash frozen and stored at -80°C until further analyses.

RNA-sequencing and data analysis

Fresh frozen tumor tissues were subsequently preserved in RNAlater (Invitrogen) and RNA was extracted using the RNeasy Midi Kit (Qiagen). After RiboGreen quantification and quality control by Agilent BioAnalyzer, 0.99-1µg of total RNA underwent ribosomal depletion and library preparation using the TruSeq Stranded Total RNA LT Kit (Illumina catalog # RS-122-1202) according to instructions provided by the manufacturer with 8 cycles of PCR. Samples were barcoded and run on a HiSeq 4000 in a 100bp/100bp paired end run, using the HiSeq 3000/4000 SBS Kit (Illumina). The output data (FASTQ files) were mapped to the human genome, converted to BAM format and expression count matrix

generated from the mapped reads using HTSeq (https://htseq.readthedocs.io/en/release_0.11.1/). Read counts were normalized for individual transcript lengths, log transformed, and differential gene expression analysis was performed using the R package DeSeq2. Significant results were filtered by adjusted p-value <0.05 and mean expression >100 reads. Significantly differentially expressed genes were subjected to pathway analysis using the GSEA software (31). The Broad Institute's Molecular Signature Databases (MSigDB) was used to retrieve significant pathways listed in the curated (C2) KEGG pathway, and hallmark (H) gene sets (32). Enrichment plots were generated using GSEA 4.1.0 for all significant pathways, determined by FDR q-value (<0.25) as suggested by GSEA for exploratory discovery. The matrix and raw data for RNA-seq reported in this paper have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE14476.

Results

Functionally disruptive NER pathway gene mutations may be present in a significant subset of patients with cancer

The Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay is an FDA authorized diagnostic test designed to detect somatic and germline alterations in over 400 cancer-associated genes (21). We leveraged the prospective MSK-IMPACT initiative to define the spectrum of NER gene mutations in a cohort of over 40,000 patients with advanced/metastatic cancers. Somatic mutational data were available for all patients whereas fully curated sample-matched germline mutational data were available for ~20,000 patients. NER pathway members, *ERCC2*, *ERCC3*, *ERCC4* and *ERCC5* are included in our sequencing panel. These four proteins, together with ERCC1 (which forms a heteroduplex with ERCC4) are major pleiotropic NER factors. Another reason to include *ERCC4* and *ERCC5* in this analysis was that both earlier studies as well as a new report carried out with the irifolven precursor drug illudin S indicate that *ERCC1/4* and *ERCC5* deficiency can sensitize to this class of drugs as well (16, 33).

We filtered all observed variants within these four genes to generate final tumor and normal datasets including only variants predicted to impair gene function. *ERCC3* p.R109X, a hypomorphic mutation, is one of the most common germline variants in the NER pathway and was observed across a range of different cancer types. As expected, missense variants made up the largest part of the alterations detected. There is evidence from both hereditary syndromes involving *ERCC2-5* and functional studies mainly interrogating *ERCC2* missense variants that a significant fraction of these variants may impair gene function. Therefore, we decided to include missense variants in our analysis, but applied several filtering methods to exclude the ones unlikely to affect gene function. Filtering steps are depicted in Figure 1; briefly we excluded intronic and synonymous variants as well as variants with a minor allele frequency of more than one percent. To predict pathogenicity within the remaining set of variants we leveraged various databases and in-silico predictions to exclude variants predicted to be benign/tolerated (for detailed description see methods section). These, together with high confidence pathogenic/likely pathogenic (P/LP) variants which include truncating (nonsense and frameshift), canonical splice site and start-loss/stop-

loss variants, were included in our final dataset as shown in Figure 1. All variants with additional information and pathogenicity prediction scores are included in Supplementary Table S2 (germline dataset) and Supplementary Table S3 (somatic dataset). Together with predicted LP missense variants putative pathogenic germline alterations in the NER pathway genes *ERCC2*, *ERCC3*, *ERCC4* and *ERCC5* were seen at a frequency of about 5-10% within all cancer types examined with the highest overall mutation burden observed in *ERCC2* (Fig. 1A). For a more conservative estimate we created an additional subset of variants, including high confidence P/LP variants and only missense variants that are linked to development of NER-deficiency syndromes in the homozygous/compound heterozygous state or ones that have been functionally validated (11, 15, 34, 35). These variants were observed in 2.3% of patients on average across cancer types (0.98-3.81%; Supplementary Table S4).

Within the MSK-IMPACT tumor dataset, we identified >1,200 patients whose tumors had potentially disruptive somatic alterations (mutations and deletions) in *ERCC2-5* (Fig. 1B). Notably, somatic (combined P/LP and candidate LP missense) mutations and deletions of *ERCC2-5* were found in ~10% of patients each with bladder and uterine cancers, and >5% of patients with bowel or skin cancers (Fig. 1B). Highest confidence P/LP variants, deletions as well as NER-deficiency syndrome associated and functionally validated missense variants were observed in 1.3% of patients on average across cancer types (0.17-5.3%; Supplementary Table S5). While loss of heterozygosity (LOH) in the tumor was observed in 3 cases with germline mutations of *ERCC3*, overall, the rate of LOH was not significantly different in *ERCC2-5* mutation carriers compared to non-carriers across 30,000 patients for whom LOH data was available (Supplementary Table S6). Additionally, in a subset of patients with truncating *ERCC3* germline variants, only 6.5% showed a second somatic deleterious alteration in a NER pathway gene (*ERCC2-5*). In sum, somatic gene mutations that could impair NER pathway function were found in a subset of patient's tumors, and thus effective precision therapies targeting vulnerabilities in NER deficient tumors could have significant clinical impact on this patient population.

Cells carrying a hypomorphic *ERCC3* mutation show significant sensitivity to irifolven

To delineate the functional effects of NER pathway mutations and to explore agents as potential therapeutic drugs that can exploit NER deficiency we generated isogenic cellular models carrying specific hypomorphic mutations identified in our cancer patient cohort. We had previously engineered non-transformed human mammary epithelial (HMLE) cells to harbor a heterozygous (c.325 C>T, p.R109*) *ERCC3* mutation using CRISPR/Cas9 based homology directed repair (14). We used the isogenic cell line pair to identify compounds with increased toxicity to the *ERCC3* mutant cells. We initially tested a range of compounds, that have either been shown to be active in various DNA repair deficient cells and organisms (cisplatin, melphalan, mitomycin C, olaparib and irifolven) or compounds previously described to affect *ERCC3* function (triptolide and spironolactone). Among these seven compounds, the most significant dose-dependent difference in sensitivity between the *ERCC3* wildtype and R109X heterozygous mutant cells were observed with irifolven (Fig. 2A) and spironolactone (Fig. 2G). Strikingly, at the lowest concentration of irifolven tested (150nM), wild-type cells showed no reduction in viability, whereas the cell viability of the

R109X mutant cells was reduced by almost 40% (IC₅₀_{WT}: 370 nM, IC₅₀_{MUT}: 160 nM). A small but significant increase in sensitivity of the mutant cell line was observed in response to cisplatin (Fig. 2B; IC₅₀_{WT}: 6.8 μM, IC₅₀_{MUT}: 5.3 μM) and mitomycin C (Fig. 2C; IC₅₀_{WT}: 363 nM, IC₅₀_{MUT}: 216 nM) but compared to irifolven the overall difference was small and/or restricted to a narrow dose-range. No significant differences in sensitivity between *ERCC3* wildtype and mutant cells were observed using the bifunctional alkylating agent melphalan (Fig. 2D; IC₅₀_{WT}: 5.9 μM, IC₅₀_{MUT}: 5.5 μM) and the Poly (ADP-ribose) polymerase (PARP) inhibitor olaparib (Fig. 2E; IC₅₀_{WT}: 39.2 μM, IC₅₀_{MUT}: 37.5 μM). Triptolide, a diterpenoid epoxide with anti-inflammatory and immunosuppressive activities, has been shown to covalently bind human *ERCC3* and inhibit its DNA-dependent ATPase activity (36). We found that triptolide treatment significantly lowered *ERCC3* transcript levels (Supplementary Fig. S2), but not protein levels in both wildtype and R109X mutant cells (Fig. 2H) and showed only mild differential toxicity in the isogenic cell line pair (Fig. 2F; IC₅₀_{WT}: 11 μM, IC₅₀_{MUT}: 7.4 μM). Spironolactone, a widely used mineralocorticoid receptor antagonist has been described to induce E1 ubiquitin ligase mediated degradation of *ERCC3*. We observed that R109X mutant cells were more sensitive than *ERCC3* wildtype cells to spironolactone in vitro (Fig. 2g; IC₅₀_{WT}: 35.4 μM, IC₅₀_{MUT}: 14.5 μM) and confirmed loss of *ERCC3* expression two hours after spironolactone treatment (Fig. 2H). Since baseline levels of *ERCC3* are significantly lower in the *ERCC3* R109X mutant cells compared to WT cells, they are depleted more rapidly of this essential protein when exposed to spironolactone. However, in contrast to irifolven, prolonged spironolactone exposure was required to exert its effect *in vitro* (Supplementary Fig. S3a). We also did not detect increased *in vivo* efficacy of spironolactone as a single agent in *ERCC3* R109X mutant xenografts at doses considered safe for use in mice (Supplementary Fig. S3b). Thus, spironolactone is unlikely to be clinically useful as a targeted anti-cancer therapy in NER deficient cells.

Truncating and missense mutations in *ERCC2* confer strong sensitivity to irifolven

To interrogate response of additional NER pathway genes to irifolven, we generated CRISPR/Cas9 edited human mammary epithelial (HMLE) cell lines harboring specific somatic heterozygous (c.237 G>T; p.E79D) or homozygous (c.237 G>T; p.E79D and c.71 A>G; p.Y24C) missense variants in *ERCC2* (Supplementary Fig. S4). Both mutations were observed multiple times within our patient cohort and are absent from gnomAD. In addition, an isogenic human bladder cancer cell line (KU1919) pair was used, since somatic *ERCC2* mutations are most common in bladder cancers (37). The KU1919 mutant cell line was engineered to harbor a compound heterozygous *ERCC2* mutation (c.1451 C>A/c.1452insG; p.T484K/L485fs*15 and c.1449delACGCTG; p.T484_L485del). The *ERCC2* mutant KU1919 cells showed reduced proliferation as compared to wildtype cells, which was not observed in the HMLE *ERCC2/3* mutant cells (Supplementary Fig. S5). When comparing drug sensitivities in the KU1919 cell line pair, the *ERCC2* mutant cells showed greater sensitivity to both irifolven and cisplatin (Fig. 2I, L). Strikingly, irifolven (IC₅₀_{WT}: 399 nM, IC₅₀_{MUT}: 98 nM) conferred a stronger selective toxicity against the mutant cells as compared to cisplatin (IC₅₀_{WT}: 3.2 μM, IC₅₀_{MUT}: 1 μM). Especially in the lower dose range (IC₅₀ and below), concentrations at which the wildtype cells were relatively unaffected, irifolven significantly [p-value <0.0001; Figure 2] reduced growth of the mutant

cells. At the wildtype IC₂₅ concentration (a concentration that reduced viability of wildtype cells by 25%), irofulven inhibited the growth of the mutant cells by about 90%, whereas cisplatin leads to less than 70% reduction in cell number. Moreover, irofulven displayed higher potency, with anti-tumor effect observed in the nanomolar range compared to cisplatin which exerted cytotoxic effects at micromolar concentrations. HMLE cells expressing the *ERCC2* c.237 G>T (p.E79D) missense variant were strongly sensitive to irofulven (IC₅₀_{WT}: 373 nM, IC₅₀_{HET}: 238 nM, IC₅₀_{HOM}: 16 nM) and cisplatin (IC₅₀_{WT}: 7.2 μM, IC₅₀_{HET}: 4.4 μM, IC₅₀_{HOM}: 0.65 μM) with a stronger differential toxicity to irofulven again observed in mutant cells (Fig. 2J, M). Here, the wildtype IC₂₅ dose decreased the number of E79D heterozygous mutant cells by more than 50% (irofulven) as compared to less than 40% (cisplatin). Similarly, the homozygous *ERCC2* c.71 A>G (p.Y24C) variant was highly sensitive to both irofulven and cisplatin (Fig. 2K, N). When comparing the two *ERCC2* mutants, the *ERCC2*E79D mutation seemed to more strongly perturb *ERCC2* function, as indicated by increased drug sensitivity in comparison to the Y24C mutant cells. For both irofulven and cisplatin the IC₅₀ concentration for the homozygous E79D cells was significantly lower than that required to achieve a 50% reduction in cell viability of the homozygous Y24C cell line (IC₅₀_{IRO}=139 nM, IC₅₀_{CIS}=1.86 μM).

ERCC2 and ERCC3 mutant cells have impaired ability to repair irofulven induced DNA damage

To assess DNA damage levels and biochemical response to irofulven treatment, we determined expression of DNA damage and cell cycle checkpoint markers via western blotting. During continuous exposure to a sub-lethal dose of irofulven (HMLE: 150nM, KU1919: 100nM) over time both *ERCC2* and *ERCC3* mutant cell lines exhibited greater baseline levels of DNA damage as indicated by increased phosphorylation of H2AX in untreated cells (Fig. 3). The engineered HMLE mutant cells showed stronger activation of DNA damage and checkpoint signaling after different durations of exposure to irofulven (Fig. 3A). This effect was observed in both the heterozygous and homozygous *ERCC2* E79D mutant HMLE cell lines as well (Fig. 3B). The parental *ERCC2* wildtype KU1919 bladder cancer cell line was resistant to irofulven and showed no substantial increase in DNA damage even with prolonged exposure to irofulven, whereas the isogenic *ERCC2*-deficient KU1919 cells demonstrated significantly increased DNA damage and p53 activation after prolonged treatment with irofulven (Fig. 3C). DNA damage and was examined by flow cytometry at various timepoints after a two-hour exposure to irofulven (HMLE: 150nM, KU1919: 100nM) and subsequent recovery after removal of the drug (Fig. 3D-E and Supplementary Fig. S6). In the non-transformed HMLE cells, we observed an initial increase in γ-H2AX that was similar in both wildtype and *ERCC2/3* mutant cells, but a reduction in this marker of DNA damage was delayed in *ERCC2/3* mutant cells. This effect was most pronounced at 18 hours post treatment when wildtype cells showed a more than 50% reduction of γ-H2AX, but *ERCC2/3* mutants retained more than 70% of γ-H2AX (Fig. 3D, E). In the bladder cancer cells, short exposure with a sub-IC₅₀ dose of irofulven did not robustly induce H2AX phosphorylation in the wildtype background, whereas the *ERCC2* mutant cell line showed a maximal increase in γ-H2AX levels 12 hours after the drug was removed from the cells that did not return to baseline levels by 24 hours post drug

exposure (Fig. 3F). In addition to impaired removal of γ -H2AX we also observed impaired recovery of RNA synthesis in the *ERCC2* mutant cells and differences in RNA Polymerase II dynamics in both *ERCC2* and *ERCC3* mutant cell lines upon irofulven treatment (Supplementary Figure S7).

Irofulven significantly inhibits the growth of ERCC2 and ERCC3 mutant tumors

To assess response of NER mutants to irofulven *in vivo* in both naturally arisen and genetically defined tumors, we used a PDX model as well as isogenic cell line derived xenografts. A PDX model from a patient with small cell lung cancer was used, harboring a heterozygous truncating mutation in *ERCC2* p.E85* (c.253G>T). We used a previously established dose of 3.5mg/kg irofulven that did not lead to sustained weight loss in mice when administered consecutively for five days (Supplementary Fig. S8). Cisplatin was used at the same dose of 3.5mg/kg and administered once per week to avoid renal toxicity. Both irofulven and cisplatin treatment suppressed tumor growth over the first two treatment cycles, while after the third cycle tumors in the cisplatin treated tumors progressed (mean tumor volume: 2,065mm³) and irofulven treated tumors showed sustained growth suppression (mean tumor volume: 496mm³; Fig. 4A). To establish an *ERCC3* mutant mouse model with which we could study the anti-tumor effects of irofulven *in vivo*, as HMLE cells do not form tumors in mice, the parental and *ERCC3*R109X isogenic cells were modified to overexpress an oncogenic HRAS mutant (G12V). Tumor volumes in the vehicle treated mice measured over the course of the experiment showed no significant difference in the ability of the two cell lines to form tumors (Fig. 4B). The R109X tumors initially grew slower but reached larger tumor volumes by the time of sacrifice. Mice receiving irofulven exhibited initial tumor growth suppression in both the wildtype and mutant groups after the first round of treatment. During the second treatment cycle, tumors in the *ERCC3* wild-type group exhibited an average tumor volume significantly larger than mutant tumors (WT_{IRO}=190.4mm³, R109X_{IRO}=23.9mm³; p=0.0027). At the start of the third treatment cycle, three mice within the R109X tumor group had a complete response with the remaining mice still exhibiting stable growth suppression. By contrast, wild-type tumors resumed growth at a similar rate as initially observed in the vehicle group. At time of sacrifice, most mice treated with irofulven in the wild-type group showed progression of disease (n=7; 78%), two mice (22%) showed partial responses and no complete responses were observed (Fig. 4B). In contrast, in the *ERCC3*R109X group, we observed only one case of stable disease (10%), six partial responses (60%) and in three cases (30%), mice showed complete tumor regression.

RNA sequencing identifies a network of potentially irofulven-sensitive genes and provides insights into co-dependencies

RNA sequencing was performed on eight tumors each from the *ERCC3* wildtype and R109X vehicle-treated groups and seven tumors each from the irofulven-treated groups. Genes differentially expressed between groups were identified and subsequently overrepresented pathways were determined using the KEGG pathway and Hallmark gene sets from The Molecular Signatures Database (Supplementary Table S7). First, we compared transcriptomic profiles between the wildtype and R109X vehicle groups to assess the effect of the R109X mutation alone on global transcript expression (Fig. 5A and Supplementary

Fig. S9). *ERCC3*R109X mutant tumors showed a significantly increased expression of transcripts involved in major DNA repair pathways, most significantly in base excision repair (BER) and mismatch repair (MMR). In addition, we observed increased expression of genes involved in DNA replication, metabolism, cell cycle checkpoint signaling and the proteasome pathway. Among the most significantly down-regulated pathways were the TGF- β pathway and protein secretion, the latter indicating increased ER stress. Next, we compared each of the vehicle groups to their respective irifolven treated groups. In the wildtype background, irifolven treatment led to a significant increase in expression of genes involved in DNA repair pathways, especially BER and homologous recombination (HR), as well as DNA replication, metabolism and cell cycle checkpoint signaling (Fig. 5B and Supplementary Fig. S10). These pathways already showed a strong upregulation in the R109X mutant tumors even without irifolven exposure and no significant increase in these pathways was observed in the R109X tumors following treatment with irifolven (Fig. 5C and Supplementary Fig. S11). Instead, in the mutant background irifolven treatment resulted in increased expression of genes involved in pathways including the p53/apoptosis pathway, TGF- β and mitogen-activated protein kinase (MAPK) signaling pathways and ribosomal biogenesis. Pathways significantly downregulated following irifolven treatment included protein secretion in both *ERCC3* wildtype and mutant groups and cell cycle checkpoint signaling specifically in the R109X mutant tumors. De-regulation of the immune network was seen in both wildtype and mutant tumors following irifolven treatment. Interestingly, irifolven treatment had the opposite effect on the MAPK signaling pathway depending on the genotype, with wildtype tumors showing downregulation of KRAS signaling while this pathway was up-regulated in the mutant tumors following irifolven treatment.

Irifolven acts synergistically with PARP inhibition, platinum and inhibition of ER-associated degradation

Based on our observation that tumors treated with irifolven showed an upregulation of genes related to DNA repair pathways other than NER, we decided to examine the effect of irifolven in combination with the PARP inhibitor olaparib. We found that the two compounds acted synergistically as defined by the Chou-Talalay method (38) over a broad concentration range in *ERCC3* mutant cells (Combination Index (CI)=0.46-0.84) and showed synergistic to additive effects in wildtype cells (CI=0.43-1.1) (Fig. 6A and Supplementary Table S8). The combination of irifolven and cisplatin acted in a weakly synergistic to additive manner in both wildtype (CI=0.84-1) and mutant (CI=0.93-1.1) cells (Fig. 6B and Supplementary Table S9). The combination of irifolven with an inhibitor of ER-associated protein degradation (ERAD), eeyarestatin I, showed synergistic activity across a broad dose-range in both wildtype (CI=0.71-0.94) and mutant (CI=0.71-0.92) cells (Fig. 6C and Supplementary Table S10). In bladder cancer cells, we found the combinations of irifolven and cisplatin to act synergistically. When using the Chou-Talalay method, synergism is observed specifically in *ERCC2* mutants, while the combination is determined to have an additive effect in wildtype cells. In addition, we used the Loewe score to determine and display synergy over the wide range of combinations used in a 3-dimensional model, which determines overall synergistic activity of cisplatin and irifolven in both cell

lines, with a higher synergy score for the *ERCC2* mutant cell line (Fig. 6D and Supplementary Table S11).

Discussion

The occurrence and impact of alterations in the nucleotide excision repair (NER) pathway on cancer risk have recently been described, and we and others have shown that both germline and somatic mutations in NER pathway genes are observed in patients with cancer across multiple cancer types (21, 39-42). Currently, platinum constitutes the preferred therapy for patients with urothelial tumors bearing mutations in *ERCC2* (15, 43). Here, we demonstrate that another chemotherapeutic agent, irifolven, could constitute a precision therapeutic against tumors of diverse types but bearing shared genetic defects in NER pathways. Irifolven shows high selective toxicity in cells and tumors with hypomorphic mutations in the nucleotide excision repair genes *ERCC2* and *ERCC3*, surpassing effects of currently used chemotherapeutics, while exhibiting lower toxicities to wildtype cells. Our results are consistent with prior studies indicating vulnerability of *ERCC2/3* deficient cells to precursors of irifolven (16). Early studies were performed on rodent cell lines harboring chemically induced biallelic frameshift mutations. The current findings build upon those early observations and significantly extend their impact towards the larger number of patients with cancer, observed to harbor putatively damaging monoallelic NER gene mutations. Experiments in human normal mammary epithelial and bladder carcinoma cell lines engineered to carry clinically observed heterozygous NER mutations, as well as relevant tumor models show that those mutations, in different cellular backgrounds, conferred selective sensitivity to this class of drugs *in vitro* and *in vivo*. Our results are in line with a separate, non-overlapping study by Börcsök et al. (co-submitted) demonstrating significant irifolven sensitivity in a set of *in vitro* and *in vivo* models with deficiencies in common NER or TC-NER factors. Importantly, Börcsök et al. also show that NER mutant cells with acquired resistance to cisplatin retain sensitivity to irifolven which would make irifolven an attractive candidate therapeutic even for patients that demonstrate acquired resistance to platinum therapy. Irifolven, and other sesquiterpenes, have been reported to act through a unique mechanism of action, inducing a type of DNA damage that requires an intact TC-NER pathway to be resolved (20, 44). Irifolven has also been shown to exhibit potent antitumor activity across a range of cancer cell lines, including those with multi-drug resistant phenotypes as well as cell lines resistant to platinum and taxane drugs (45). Because of these properties, between 1999-2006 multiple clinical trials evaluated the efficacy of irifolven among genotypically unselected patients with various tumor types; generally mixed responses and some reports of early toxicities in these trials limited subsequent investigations (46). While the overall response rate in treated patients in these historical trials was not superior to comparison drugs, a small subset of patients showed substantial responses following irifolven. Based on our observations in isogenic cell line and xenograft models, we predict that patients harboring mutations in *ERCC2* or *ERCC3* will demonstrate a high probability of response to irifolven and related compounds.

Analysis of a large cohort of patients with cancer showed that *ERCC2* and *ERCC3*, as well as *ERCC4* and *ERCC5*, encoding for NER-specific helicases and incision nucleases, are mutated both somatically and in the germline of patients with cancer, thus pointing to a

subset of patients that could benefit from irifolven or irifolven-analog therapy. Since *ERCC2* somatic mutations already constitute a biomarker for bladder cancer treatment with platinum drugs, irifolven may constitute a precision drug option for a subset of patients, specifically cisplatin non-responders. Irifolven may also provide a potential first line of treatment in this and other tumor contexts, since it showed high activity in NER pathway mutant mammary as well as bladder cancer- derived cells and in xenograft models, but reduced toxicity in wildtype cells compared to cisplatin.

Mechanistically, it has been shown that acylfulvenes alkylate DNA and form DNA adducts that disrupt DNA and RNA synthesis, arrest cells in G1-S phase, and induce apoptosis (47). These findings are in agreement with our transcriptome analysis showing an increase in expression of genes involved in those pathways following irifolven treatment. The increase in cell cycle checkpoint signaling already observed in untreated *ERCC3* mutant cells warrants further exploration. Our observation that the TGF- β pathway is down-regulated in the R109X mutant cells is consistent with a recent study showing decreased TGF- β signaling in NER-deficient cells (48). Our data also suggest the potential for using irifolven in combination with PARP inhibitor treatment, a synergism that could lead to an improved response. Both *in vitro* drug combination experiments as well as RNA-seq data results point to dependence of NER mutants on other repair pathways, especially base excision repair. It is important to note here that the six major DNA repair pathways in humans share common proteins and work closely together to resolve complex lesions and maintain genome integrity. A stronger than previously appreciated cooperation has recently been described between NER and BER in the repair of various types of lesions (49). The HR and non-homologous end joining (NHEJ) pathways are activated as a result of both direct and indirectly formed DNA double strand breaks, the latter resulting when DNA insults cannot be resolved by the other DNA repair mechanisms. Due to high cooperativity between DNA repair pathways alterations in a pathway other than the one targeted by a specific drug can lead to resistance. This has been shown in the case of PARP inhibition (PARPi) where resistance is often caused by *BRCA1/2* reversion mutations but can also arise due to loss of NHEJ pathway activity (50). Similarly, increased activation of either the NER, HR or MMR pathways have been associated with cisplatin resistance (51). Overall this indicates that combinations of agents targeting different DNA repair pathways could be very efficient and help to overcome drug resistance. Combinations of PARPi and platinum agents have been explored in multiple Phase I-III clinical trials albeit with limited success since overlapping toxicities required dosing of both agents to be reduced (52). Since these trials were not performed on patient cohorts selected for biomarkers of tumor DNA repair deficiency in which even a combination of these drugs at reduced doses could generate successful results, we propose testing combinations such as PARPi and irifolven in PDX models from NER deficient patients and – if successful – in subsequent precision medicine combination trials such as NCI-ComboMATCH. Such studies can provide further evidence of the efficacy of targeted combination therapy in a specific genetic background. Our RNA-seq data also suggests an increased dependence of *ERCC3* mutant cells on the unfolded protein response (UPR) pathway, as indicated by a significant reduction in protein secretion, which is augmented in the presence of irifolven. Our *in vitro* combination experiments have also shown synergistic activity of an irifolven combination with ERAD inhibition, in both

ERCC3 wildtype and mutant cells. Thus, overall, our drug combination experiments indicate that the success of this therapeutic strategy will depend on both cellular as well as specific genetic background.

The observed perturbed DNA repair ability of *ERCC2* and *ERCC3* heterozygous mutant cells further indicates that these pathogenic variants, present in the germline or in tumors absent homozygous mutations or loss of heterozygosity, act as hypomorphs. We have previously suggested that heterozygous *ERCC3* mutant cells, like other components of DNA damage repair pathways (e.g. H2AX, BLM, CHEK1), may act via dosage dependency and mechanisms such as haploinsufficiency, leading to tumorigenesis (14). The current data suggest a model whereby the resulting functional impairment does not appear to affect basal transcription but affects DNA repair under conditions of prolonged or increased genotoxic stress. Such prolonged stress may give rise to additional oncogenic mutations leading to cancer formation as well as increased intra-tumoral genetic instability.

Overall, we identified many patients with cancer that harbor alterations in NER core pathway members *ERCC2-5*. Although the NER pathway consists of more than 40 members, *ERCC2-5* genes are the major pleiotropic members of the pathway and most frequently altered in syndromic patients (11). As we observe similar rates of LOH among NER-proficient and deficient patients, we would not assume that alterations in these genes act as classical tumor suppressors. Instead, mutations in NER pathways may play a role in the initial steps of cancer formation but may act in concert with other deficiencies. While we observe both low rates of LOH and low incidence of co-occurrence of somatic NER gene mutations in germline carriers of pathogenic NER variants, more than a third of tumors show co-occurrence of pathogenic NER germline variants with likely pathogenic alterations in other DNA repair pathway genes which could cooperate in generating overall genomic instability. In addition, complete inactivation of *ERCC2/3* may be detrimental to tumor growth based on their involvement in general transcription as members of the TFIIH complex. While the mechanism of NER gene involvement in tumor initiation/progression requires further investigation, alterations affecting function of these genes appears to sensitize tumors to NER targeting agents. Patients with both germline and somatic LoF variants would qualify for treatment with NER targeting agents, but prior to treatment of germline mutation carriers safety studies should be performed using mouse models harboring heterozygous germline mutations in NER genes. Although increased general toxicities have not been observed with PARPi treatment of *BRCA1/2* germline carriers, the effect of irifolven has not been studied yet in this context.

Limitations in the current study include the definition of NER deficiency in the patient population by applying a predictive model in which putative pathogenic mutations are classified largely through *in silico* prediction tools. Especially for missense variants, additional functional models will be needed to classify candidate variants. However, missense variants in the core NER genes *ERCC2-5* are frequently reported among patients with hereditary syndromes; in the case of *ERCC2*, functional studies have shown that many of these missense variants are deleterious. After applying several filtering steps for increased stringency, selected missense variants were reported, including the ones reported in the *in vitro* experiments here. The resulting data suggests that a defined subset of patients could

benefit from treatment exploiting NER deficiency, a hypothesis that needs further exploration and validation. Such validation will require additional large datasets across tumor types as well as expanding the assessment of irifolven sensitivity to NER pathway members other than *ERCC2* and *ERCC3*. To identify potential candidates for a “basket” type clinical trial, orthogonal approaches to determine NER deficiency, such as tumor profile or functional assays would be helpful. In addition, our current studies have been done on a limited set of *in vitro* and *in vivo* models and may not be transferrable to all types of mutations and tissues. Therefore, it will be of interest to assess a larger panel of PDX models derived from different tissue types and NER pathway deficiencies. Although multiple clinical trials were initiated with irifolven, including one at our institution in the early 2000s (NCT00005070), tumor tissues from these trials were not available to retrospectively assess presence of NER pathway alterations in extreme responders. Finally, while cross-resistance between irifolven and cisplatin has not been observed, we cannot fully exclude the possibility of such cross-resistance occurring in a human clinical setting or with other DNA repair targeting agents.

To define novel treatment strategies for patients with hypomorphic mutations in the *ERCC2/3* complex, we propose a drug repurposing approach for irifolven, ideally in the form of a basket trial for patients showing NER pathway deficiency (53) for which patients can be selected in real-time based on assessment of NER deficiencies through next-generation gene panel sequencing or combination of tissue microarray (TMA) and immunohistochemistry (IHC) on biopsy or resection material. An additional strategy would be high throughput screening for other compounds that selectively target NER pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

In this study, we identify a subset of patients with cancer harboring germline or somatic aberrations within the nucleotide excision repair (NER) pathway, which has not been previously characterized. There are currently no approved NER specific therapeutic strategies that target this group of patients. We demonstrate that recurrent heterozygous mutations in NER pathway genes *ERCC2* and *ERCC3*, as observed in these patients, can confer significant sensitivity *in vitro* and *in vivo* to the previously developed anti-cancer drug irifolven. The present study provides a molecularly targeted, pre-clinical approach to cancers with mutations in nucleotide excision repair pathway genes demonstrating preferential sensitivity to the drug irifolven alone, or in combination with other agents. Similar to the impact of PARP inhibitors on the landscape of treatment for HR-deficient tumors, irifolven and related compounds could significantly improve treatment outcomes for patients with NER-deficient tumors.

Fig. 1: Putative functionally significant alterations in NER genes *ERCC2-5* are found in a significant subset of patients with cancer.

(A) Germline variant filtering scheme and number of patients with likely damaging *ERCC2-5* germline mutations as well as alteration frequencies across different cancer types among 16,712 patients with cancer sequenced through MSK-IMPACT. (B) Somatic variant filtering process and number of patients with likely pathogenic *ERCC2-5* somatic mutations or deletions as well as alteration frequencies across different cancer types in >40,000 patients with cancer sequenced through MSK-IMPACT. Highest frequency of mutations in those NER pathway members is observed in bowel, skin, uterine and bladder cancers.

Fig. 2: *ERCC3* and *ERCC2* mutant cells show significant sensitivity to iriofulven treatment. (A) Cells harboring the heterozygous *ERCC3* R109X mutation show significantly higher sensitivity to iriofulven compared to WT cells. (B-D) Sensitivity to other DNA crosslinking agents is only marginally increased in the R109X mutant. (E) No difference was observed in response to olaparib. (F, G) The effect of drugs previously shown to directly affect *ERCC3* was assessed, showing a small differential response for triptolide (F; inhibitor of *ERCC3* DNA-dependent ATPase activity) while a substantial differential response was observed after treatment with Spironolactone (G; inducing proteasomal degradation of *ERCC3*). (H) Western blot showing the effect of iriofulven, spironolactone and triptolide on *ERCC3* protein levels. Only spironolactone affects *ERCC3* protein levels, leading to a strong reduction at doses as low as 1μM in the mutant cells. (I, L) Bladder cancer cells harboring a compound heterozygous *ERCC2* mutation show significantly higher sensitivity to iriofulven (I) and cisplatin (L) compared to WT cells. (J, K, M, N) Human mammary epithelial cells harboring *ERCC2* missense variants E79D (J, M) or Y24C (K, N) show significantly higher sensitivity to iriofulven compared to WT cells. For the E79D mutation significance is indicated only for the comparison between wildtype and heterozygous mutant, the difference between wildtype and homozygous mutant cells are highly significant (p 0.0001) at all doses tested. Data represents the mean of three experiments with error bars representing the

SEM. Significance was determined by two-way ANOVA test. *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001

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Fig. 3: NER mutant cells treated with irofulven show increased DNA damage and impaired DNA repair kinetics.

(A-C) Induction of DNA damage and checkpoint activation in response to irofulven. Levels of H2AX, p-Chk-1 and ERCC2/3 were assessed in isogenic cell line pairs cells treated over different length of exposure to a dose of 150nM (HMLE) or 100nM (KU1919) irofulven. Western blots show increased activation of H2AX and Chk-1 in *ERCC2/3* mutant cells. The 48-hour timepoint for the E79D homozygous mutant is missing due to extensive cell death at this stage.

(D-F) Recovery from irofulven-induced DNA damage over time. Levels of γ -H2AX were assessed by Flow cytometry. *ERCC2/3* mutant cells show reduced reduction of γ -H2AX positive cells over time indicating a decrease in DNA repair efficiency. Bar graphs show average from two biological replicate experiments recording a minimum of 20,000 cells per condition. Significance was determined by two-way ANOVA test to measure effect in mutant cells compared to wildtype cells at all time points; *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001

Fig. 4: Growth suppression of *ERCC2* and *ERCC3* mutant tumors in response to irifolven and discovery of pathways de-regulated in *ERCC3* mutant tumors.

(A) Patient-derived xenografts from an individual with small cell lung cancer harboring a truncating mutation in *ERCC2*, p.E85* (c.253G>T), show substantial growth suppression in response to irifolven. Tumors initially respond to treatment with cisplatin as well, but here tumors ceased to respond after the second treatment cycle (B) Irofulven mediates strongly increased and sustained growth suppression in CRISPR engineered *ERCC3* heterozygous mutant xenografts.

Fig. 5: Pathway analysis of differentially expressed genes.

(A) Significantly Up- and Down-regulated pathways within KEGG and Hallmark gene sets in xenografts from vehicle-treated ERCC3 WT/R109X compared to vehicle-treated wildtype mice (B) Significantly Up- and Down-regulated pathways within KEGG and Hallmark gene sets in wildtype xenografts from mice treated with irifolven compared to wildtype tumors from vehicle treated mice (C) Significantly Up- and Down-regulated pathways within KEGG and Hallmark gene sets in ERCC3 WT/R109X tumors from irifolven-treated mice compared to ERCC3 WT/R109X tumors from vehicle treated mice. Red arrows mark pathways related to DNA repair signaling. Normalized enrichment scores (NES) reflects the degree to which a gene set is overrepresented at the extremes (top or bottom) across a list of genes ranked by hypergeometrical score (HGS) based on differential gene expression. Higher enrichment scores indicate a shift of genes belonging to certain pathway categories towards either end of the ranked list, representing up or down regulation (positive or negative values, respectively). Pathways with FDR q-value (<0.25) were further considered for biological relevance.

Fig. 6: Drug combinations with potential synergistic activity in *ERCC2/3* mutants. (A) Olaparib acts synergistically in combination with irofulven specifically in the *ERCC3* mutant background. (B) Combination treatment with cisplatin increases sensitivity of both *ERCC3* mutant and wildtype cells to irofulven in an additive manner. (C) Combinations of irofulven and eeyarestatin I show moderate synergism in *ERCC3* mutant cells and moderately synergistic to additive effects in wildtype cells. Representative CI plots are shown. (D) Strong synergistic activity of irofulven and cisplatin is detected in *ERCC2* mutant bladder cancer cells and weak synergism is observed in wildtype bladder cancer cells. Representative cooperativity screens and Loewe plots are shown.