A Subset of Colorectal Cancers with Cross-Sensitivity to Olaparib and Oxaliplatin

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ABSTRACT

Purpose: Defects in the homologous recombination (HR) repair pathway are of clinical interest due to sensitivity of HR-deficient cells to PARP inhibitors. We were interested in defining PARP vulnerability in patients with metastatic colorectal cancer (mCRC) carrying *KRAS* and *BRAF* mutations who display poor prognosis, have limited therapeutic options, and represent an unmet clinical need.

Experimental Design: We tested colorectal cancer cell lines, patient-derived organoids (PDO), and patient-derived xenografts (PDX) enriched for *KRAS* and *BRAF* mutations for sensitivity to the PARP inhibitor olaparib, and the chemotherapeutic agents oxaliplatin and 5-fluorouracil (5-FU). Genomic profiles and DNA repair proficiency of colorectal cancer models were compared with pharmacologic response.

Results: Thirteen of 99 (around 13%) colorectal cancer cell lines were highly sensitive to clinically active concentrations of olaparib and displayed functional deficiency in HR. Response to PARP blockade was positively correlated with sensitivity to oxaliplatin in colorectal cancer cell lines as well as patient-derived organoids. Treatment of PDXs with olaparib impaired tumor growth and maintenance therapy with PARP blockade after initial oxaliplatin response delayed disease progression in mice.

Conclusions: These results indicate that a colorectal cancer subset characterized by poor prognosis and limited therapeutic options is vulnerable to PARP inhibition and suggest that PDO-based drug-screening assays can be used to identify patients with colorectal cancer likely to benefit from olaparib. As patients with mCRC almost invariably receive therapies based on oxaliplatin, "maintenance" treatment with PARP inhibitors warrants further clinical investigation.

Introduction

Treatment of metastatic colorectal cancer (mCRC) has improved over the last 15 years since the introduction of EGFR-targeted therapy, antiangiogenic agents, and the use of intensive triplet chemotherapy regimens based on fluoropyrimidines, oxaliplatin, and irinotecan (1).

Immune checkpoint inhibitors have been shown to induce durable responses in a subset of approximately 5% patients with mCRC that carry defective mismatch repair (MMRd) or are microsatellite unstable (MSI; refs. 2, 3). Targeted therapy trials are ongoing with promising results in subsets of molecularly selected colorectal cancers, such as *BRAF* mutant or *HER2* amplified cases (4, 5) and these targeted combinations are expected to enter clinical practice in a short time-frame. However, median overall survival in patients with mCRC has reached a plateau that ranges from 18 months, in cases of *RAS* mutant and right colonic tumors, to 42 months in *BRAF/RAS* wild-type and left-side cancers (6–8). In summary, although the overall survival of patients with mCRC has been increased by combining and fine-tuning the use of cytotoxics, targeted agents, and immunotherapy, the impact of these advances has been incremental rather than transformative.

Accordingly, there is a need to improve disease control in patients with mCRC and prolong overall survival, particularly for patients who are not eligible for targeted agents or immune therapy. Furthermore, intrinsic and acquired drug resistance, as well as the neuropathy associated with oxaliplatin-containing regimens (9), limits the duration of long-term disease control and represents a clinical problem that needs to be addressed.

Molecular profiling of large colorectal cancer datasets has revealed subsets of cases with defects in DNA repair pathways, some of which may be amenable to therapeutic targeting. Germline pathogenic variants of *BRCA1*, associated with defects in the homologous recombination (HR) repair pathway, are emerging as a risk factor for colorectal cancer (10), particularly for early onset colorectal cancer (11). Importantly, recent studies have reported that up to 15% of

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Translational Relevance

There is a need to expand therapeutic options for a significant subset of patients with mCRC who do not benefit from targeted or immune therapies. Through a comprehensive screening of a large collection of CRC cell lines enriched for *RAS/BRAF* mutations, we found that up to 13% of cases are sensitive to the PARP inhibitor olaparib. We detected pharmacologic cross-sensitivity between olaparib and oxaliplatin, which is of immediate translational relevance. Maintenance therapy with PARP blockade after initial oxaliplatin response was effective in delaying disease progression in colorectal cancer patient–derived xenografts. This study lays a rationale for the design of trials testing a maintenance therapy with PARP inhibitors in the subset of patients with mCRC with complete or partial response to prior oxaliplatin treatment.

individuals carry germline or somatic genetic defects in HR repair genes (12–14). In other tumor types such as breast and ovarian cancers, defects in the HR repair machinery, due to alterations in *BRCA* or other genes, such as *RAD51* and *PALB2*, confers the so-called "BRCAness" phenotype (15). These tumors often display sensitivity to specific DNA damaging agents including platinum compounds and PARP inhibitors (16–18).

Four PARP inhibitors are approved in slightly different clinical niches for specific malignancies carrying germline *BRCA* mutations, in previously treated patients with ovarian and breast cancer. Of these, only one is also approved for maintenance therapy in patients with ovarian, fallopian tube, and primary peritoneal cancer in remission after platinum-based therapy (18–29). Approval for use in germ-line *BRCA* mutant prostate and pancreatic cancers is expected in 2020. Data in other malignancies are either immature or controversial. The development of PARP inhibitors in colorectal cancer, alone or in combination with other cytotoxic agents, has been hampered by toxicity or the lack of patient selection (30–33). On the other hand, preclinical testing of PARP inhibitors in molecularly annotated bowel tumors has not been performed extensively. Hence, in this work we set to establish the prevalence of exquisite sensitivity to PARP inhibition in a large collection of colorectal cancer cell lines and preclinical models, with the ultimate goal of defining potential predictive markers for guiding clinical development of PARP inhibitors in bowel cancer. We decided to focus on MMR-proficient *KRAS* and *BRAF* mutant colorectal cancer, as these tumors lack effective treatments or, despite novel combined target therapies, still display dismal outcome. Our work revealed that response to olaparib in colorectal cancer cell lines was positively correlated with sensitivity to oxaliplatin treatment *in vitro*. Although olaparib sensitivity could not be pinpointed by genomic defects in *BRCA* or other HR repair genes, we found that functional assays based on detection of DNA damage response were capable of predicting vulnerability to PARP inhibition in cell lines and patient-derived models.

Materials and Methods

Cell lines and cell authentication

All colorectal cancer cell lines are part of datasets we previously characterized (34, 35). Each cell line was cultured in its specific media and conditions. All cell lines were grown at 37°C in a 5% CO$_2$ air incubator. Cell lines were routinely checked for mycoplasma contamination using the PCR Mycoplasma Detection Kit (ABM) according to the manufacturer's protocol. The genetic identity of cell lines was performed using the PowerPlex 16 HS System (Promega), through Short Tandem Repeats (STR) at 16 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO, D18S51. D3S1358, D8S1179, FGA, Penta D, Penta E, and amelogenin). Amplicons from multiplex PCRs were separated by capillary electrophoresis (3730 DNA Analyzer; Applied Biosystems) and analyzed using GeneMapperID v.3.7 software (Life Technologies).

PDX and organoid establishment

Tumor samples were obtained from patients treated at Niguarda Cancer Center (Milano, Italy). All patients provided informed written consent, samples were procured, and the study was conducted in accordance with the Declaration of Helsinki and under the approval of the local Independent Ethical Committee (protocol 194/2010). To generate patient-derived xenografts (PDX), tumor specimens were subcutaneously implanted in 7-week-old NOD-SCID mice (Charles River Laboratory). All animal procedures were approved by the Ethical Committee of the Candiolo Cancer Institute and by the Italian Ministry of Health.

Patient #1 (case HROC278; ref. 35) and patient #3 organoids were established at Candiolo Cancer Center from PDX models obtained following procedures described later. To generate organoids, PDX tissue was dissociated into single-cell suspension by mechanical dissociation using the Gentle MACS Dissociator (Miltenyi Biotec) and enzymatic degradation of the extracellular matrix using the Human Tumor Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Cell suspension was centrifuged at 400 × *g* for 5 minutes and then the pellet was resuspended with organoid basal medium (consisting of advanced DMEM/F12 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L GlutaMAX, and 10 mmol/L Hepes). Final cell suspension was filtered through a 70-μm cell strainer (Falcon) and the flow-through was centrifuged three times. At the end of the washing phase, the pellet was resuspended in Matrigel (Corning) and 50 μL of organoids-Matrigel suspension were dispensed into the center of each well of a 37°C prewarmed 24-well plate. Different densities of tumor cells were plated and left to solidify for at least 30 minutes at 37°C before ENAS or WENRAS medium was added and the cells were incubated at 37°C, 5% CO$_2$. To prepare ENAS medium, the following reagents were added to Basal medium: 1 × B27 supplement (Invitrogen), 1 × N2 supplement (Invitrogen), 1.25 mmol/L N-acetyl-cysteine (Sigma Aldrich), 10 mmol/L nicotinamide (Sigma Aldrich), 10 nmol/L gastrin (Sigma), 50 ng/mL human EGF (Life Technologies), 100 ng/mL Noggin (PeproTech), 500 nmol/L TGFb type I receptor inhibitor A83-01 (Tocris), and 10 μmol/L p38 MAPK inhibitor SB202190 (Sigma Aldrich). During the first 3 days of organoid establishment, ROCK inhibitor (10 μmol/L; Selleckchem) and GSK inhibitor (3 μmol/L, Selleckchem) were added. WENRAS medium was prepared by adding 10% RSPO1-CM (conditioned medium) and 50% WNT3a-CM to ENAS medium.

Patient #2, patient #4, and patient #5 organoids were established directly from tissue biopsy obtained at the time of surgery. Organoids from patient#2 were established at INGM (Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi", Milan, Italy), whereas organoids from patients #4 and #5 were established at Candiolo Cancer Institute. Briefly, tumor tissue was first smashed in small pieces and incubated in PBS with collagenase A (0.5 mg/mL; Roche), hyaluronidase (20 μg/mL; Sigma), and 10 μmol/L ROCK inhibitor for 30 minutes at 37°C with shaking. After incubation, 5% FBS was added and the mixture was centrifuged at 400 × *g* for 5 minutes. The pellet

was washed twice in PBS to remove debris and collagenase. At the end of the washing phase, the pellet was resuspended in Matrigel (Corning) and 50 µL of organoids-Matrigel suspension were dispensed into the center of each well of a 37°C prewarmed 24-well plate. Different densities of tumor cells were plated and left to solidify for 10 to 30 minutes at 37°C before ENAS medium was added and the cells were incubated at 37°C, 5% CO_2. Fresh medium was replaced every 2 to 3 days. Outgrowing organoids were passaged every 10 to 15 days after mechanical and enzymatic disruption. Organoid drug treatment was performed in each organoid-specific culture medium at 37°C and 5% CO_2.

Genomic DNA extraction, exome sequencing, and bioinformatic analysis

Genomic DNA samples were extracted from each cell line using ReliaPrep gDNA Tissue Miniprep System (Promega) and sent to IntegraGen (France) for exome sequencing. Data analysis was performed at the Candiolo Cancer Institute following procedures as described previously (36). All synonymous variants were filtered out and only small-nucleotide variants (SNV) with variant allele frequency >10% were subsequently annotated by SIFT and PolyPhen algorithms.

MSS/MSI analysis

The microsatellite instability (MSI) status was evaluated by using the MSI Analysis System Kit (Promega) according to manufacturer's protocol. The analysis requires a multiplex amplification of seven markers including five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). The products were analyzed by capillary electrophoresis in a single injection using ABI 3730 DNA Analyzer capillary electrophoresis system (Applied Biosystems). The results were analyzed using GeneMapper V5.0 software. Samples with instability in two or more markers were defined as MS-instable (MSI-H). Samples with no detectable alterations were defined as MS-stable (MSS).

Analysis of mutational signatures

Mutational signatures were calculated selecting the nucleotide changes of variants from the SNVs report file (36): variants present in common dbSNP version 150 or in a panel of 40 normal samples previously sequenced were excluded, and only SNVs showing a statistical significant P value (less than 0.05 calculated with a binomial test on allele count and depth), as well an allelic frequency greater or equal to 10% were selected. These variants were passed through the web application "Mutational Signatures in Cancer" (MuSiCa; ref. 37). Signature profiles were calculated using the six substitution subtypes: C>A, C>G, C>T, T>A, T>C, and T>G (referring to the pyrimidine of the mutated Watson–Crick base pair). Information on 5′ and 3′ base context to each mutated nucleotide was incorporated to generate 96 possible mutation types. The output file of MuSiCa including the contribution values of 30 signatures (38) was used to create the heatmap.

Drugs

Olaparib (AZD2281, Ku-0059436, S1060), niraparib (S2741), rucaparib (S1098), oxaliplatin (S1224), and 5-fluorouracil (5-FU; S1209) were purchased from Selleck Biochem.

Oxaliplatin and 5-FU testing

Three thousands cells/well were seeded in 100 µL complete growth medium in 96-well plastic culture plates at day 0. The following day,

serial dilutions (100 µL) of oxaliplatin (0–12.5 µmol/L) or 5-FU (0–20 µmol/L) were added. Plates were incubated at 37°C in 5% CO_2 for 6 days, after which cell viability was assessed by measuring ATP content through Cell TiterGlo Luminescent Cell Viability assay (Promega). Luminescence was measured by the SPARK M10 (Tecan) plate reader. Treated wells were normalized to untreated wells.

Olaparib testing

Olaparib sensitivity was tested in long-term proliferation assays. Briefly, cells were seeded in 24-wells plates at day 0 and the following day they were treated with serial dilutions of olaparib (0.5–15 µmol/L). The treatment was refreshed every week and the assay was stopped when untreated cells reached confluency (from 10 days to 2 weeks of treatment). Plates were fixed with 4% paraformaldehyde (Santa Cruz) and stained with 1% crystal violet-methanol solution (Sigma-Aldrich), which was then solubilized by 10% acetic acid and quantified by measuring the absorbance at 600 nm at the SPARK M10 (Tecan) plate reader. Treated wells were normalized to untreated wells.

Immunofluorescence detection of RAD51 foci

Cells were grown on glass coverslips (2×10^5 cells per well) in a 24-well plate. Twenty-four hours after plating, the cells were exposed to ionizing radiation at the indicated doses and allowed to recover for 4 hours. After recovery, the cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.1% Triton-X100 in PBS for 5 minutes. Cells were incubated at room temperature with 1% BSA in PBS for 30 minutes, followed by incubation overnight at 4°C with the following primary antibodies diluted in PBS containing 1% of BSA and 1% of donkey serum: anti-phospho-Histone H2AX (Ser139) rabbit monoclonal antibody (Bethyl Laboratories; 1:600) and anti-RAD51 rabbit polyclonal antibody (Millipore; 1:100). After washing, cells were fluorescently labeled according to the primary antibody used with an Alexa Fluor 555 donkey anti-mouse antibody or Alexa Fluor 488 donkey anti-rabbit antibody (Molecular Probes; 1:400) for 1 hour at room temperature. Nuclei were stained with DAPI. A Leica DMI6000B fluorescence microscope (Leica Microsystems) under a 40× dry objective was used to detect γ-H2AX and RAD51 foci. Images were captured at 10 individual z-planes and were merged using the "Z Project" function in ImageJ. Individual nuclei were scored for foci positivity as identified based upon signal intensity above general background staining levels and present within the nucleus as assessed by DAPI staining. Cells containing ≥5 distinct foci were defined as foci-positive, and the percentage of positive nuclei was calculated as [(number of foci positive nuclei)/(number of nuclei scored)] × 100. A minimum of 500 nuclei per sample were scored.

Generation of pDR-GFP–expressing cells

The colorectal cancer cells were seeded at 25×10^4 cells/well in six-well plastic culture plates. The following day, cells were transfected with pDR-GFP plasmid (AddGene) using Lipofectamine 3000 following manufacturer's instruction. Seventy-two hours after infection, puromycin (Sigma Aldrich) was used to select stably infected cells.

Plasmid-based assay to measure HR

The pDR-GFP–expressing cells were seeded at 25×10^4 cells/well in six-well plastic culture plates. The following day, cells were transfected with pCBASce-I expressing plasmid (AddGene) using Lipofectamine 3000 following manufacturer's instruction. Forty-eight to sixty hours after transfection, cells were harvested and analyzed by flow cytometry. The relative HR capacity was determined by dividing the percentage of

GFP positive cells in *Sce*-I transfected cells by the basal percentage of GFP signal in mock control.

Drug assays in organoids

Organoids were enzymatically dissociated into single-cell suspensions using TrypLE Express Enzyme (Thermo Fisher Scientific) for 15 to 30 minutes at 37°C. After washing, cells were counted and seeded in 2% BME/growth media (5,000–6,000 cells/well) in 96-well plates precoated by basement-membrane extract (BME; Cultrex BME Type 2; Amsbio). Depending on the organoid sample, the drug treatment started between day 2 and 10 after seeding when small organoid structures were visible. Cells were then treated with olaparib or oxaliplatin diluted in 2% BME/growth media for 18 days. Fresh 2% BME/organoid medium containing drugs was refreshed at least three times during the treatment. Organoid viability was assayed at the end of the experiment by CellTiter-Glo (Promega) according to manufacturer's instruction with modifications. Briefly, reagent was mixed 1:1 with organoid media, and organoids were then subjected to the lysis by shaking at 600 rpm for 15 minutes.

In vivo treatment

Established tumors were treated with the indicated drugs following specified schedule: olaparib: every day (from Monday to Friday), 50 mg/kg i.p. (vehicle: PBS 10% 2-hydroxy-propyl-β-cyclodextrin; Sigma-Aldrich); oxaliplatin: once weekly for a total of two treatments, 10 mg/kg i.p. (vehicle: dextrose 5% water; Sigma-Aldrich). Four to six mice were included in each experimental group. Tumor size was evaluated weekly by caliper measurements. Animal procedures were approved by the Ethical Commission of the Candiolo Cancer Institute and by the Italian Ministry of Health.

Statistical analysis

Results were expressed as means ± SEM or SD as indicated in the legend. Statistical significance was evaluated by *t* test or two-way ANOVA using GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Results

A subset of colorectal cancer cells is sensitive to olaparib

mCRCs bearing mutations in *KRAS* or *BRAF* and displaying stable microsatellite status (MSS) have dismal prognosis and are not eligible for therapies based on anti-EGFR antibodies or immune-checkpoint blockade (39, 40). To model this subset, we selected 99 colorectal cancer cell lines enriched for *KRAS* and *BRAF* mutations or other alterations conferring resistance to EGFR blockade from our extensive collection (Supplementary Table S1). All colorectal cancer lines were challenged with five concentrations of olaparib (0.5, 1, 5, 10, and 15 µmol/L). These concentrations were defined on the basis of olaparib plasma levels known to be clinically achievable and therapeutically relevant for the treatment of patients with ovarian and breast cancer (41, 42). The BRCA2 deficient pancreatic cell line (CAPAN1), which is sensitive to PARP blockade, served as positive control. Most of the tested colorectal cancer cell lines were markedly resistant across the entire range of olaparib concentrations. However, up to 13% of cell lines were sensitive at low drug concentrations at a level comparable to CAPAN1 cells (**Fig. 1**). Olaparib sensitive cell lines were also responsive to the other FDA-approved PARP inhibitors niraparib and rucaparib, underlying a likely drug class effect (Supplementary Fig. S1). When all three drugs were tested at equimolar concentrations, niraparib showed greatest potency, consistent with the previously reported higher PARP-trapping activity (43, 44).

Genomic profiles and sensitivity to olaparib

Mutations in *KRAS, NRAS,* or *BRAF* genes, which are commonly assessed as biomarkers in colorectal cancer, were not associated with sensitivity or resistance to olaparib (**Fig. 1**). Olaparib-sensitive cell lines were not enriched for any of the transcriptional consensus molecular subtypes (CMS) of colorectal cancer (45). Nevertheless, none of the olaparib-sensitive cells were classified as CMS2 (data not shown).

Considering that in breast and ovarian cancer sensitivity to PARP blockade is often associated with *BRCA1* and *BRCA2* mutations, we employed a clinically validated BRCA test (Cogentech). This analysis did not reveal mutations in either *BRCA1* or *BRCA2* previously validated to be associated with HR deficiency and sensitivity to PARPi (Supplementary Table S2). Nevertheless, *in silico* assessment of a previously unreported *BRCA2* variant in CX1 cells revealed the occurrence of a potentially pathogenic mutation, which has not yet been functionally assessed. To identify further molecular alterations underlying olaparib sensitivity, we performed whole-exome sequencing (WES) analysis on the entire cell collection. This led to the identification of mutations in genes previously functionally associated to HR deficiency in human cells (Supplementary Table S3; Supplementary Fig. S2).

HR deficiencies have been linked to distinct mutational signatures (46–48), which are combinations of nucleotide changes arising from specific mutagenesis processes, such as exposure to DNA damaging conditions and alteration in DNA replication processes (49). Over 30 mutational signatures have been identified in human cancers, a subset of which are linked to defective DNA repair pathways (49, 50). We therefore queried whether mutational signatures could discriminate olaparib-sensitive and -resistant colorectal cancers. The approach positively identified mutational profiles frequently occurring in colorectal cancer, such as signatures 6 and 10 (Supplementary Fig. S2A). Of note, 2 of 13 olaparib-sensitive cells displayed signature number three which is correlated with HR defects and BRCAness (47, 49). Overall, however, mutational signatures did not discriminate between olaparib-sensitive and -resistant colorectal cancers (Supplementary Figs. S2A and S2B; refs. 51, 52).

Functional assays differentiate olaparib-sensitive and -resistant colorectal cancer cells

We reasoned that functional assays could be used to identify olaparib-sensitive colorectal cancer cells. To this end, we performed two types of analyses: first, we used radiation to induce DNA double-strand breaks (DSB) in olaparib-sensitive and -resistant models and measured the percentage of γH2AX and RAD51 foci positive nuclei postradiation (**Fig. 2A**). The number of γH2AX foci positive nuclei increased comparably after radiation in both groups as expected (**Fig. 2A**, top; **Fig. 2B**; Supplementary Fig. S3A). Notably, a marked difference between sensitive and resistant colorectal cancer models was observed in the RAD51 assay. Upon radiation, the number of RAD51 foci positive nuclei increased in all olaparib-resistant cells, whereas the same effect was not observed in the sensitive cells (**Fig. 2A**, bottom; **Fig. 2C**; Supplementary Fig. S3B).

Next, we directly measured HR capabilities using a two plasmid-system (pDR-GFP) designed to detect HR deficiencies in human cells (53). This strategy involves stable expression of two GFP cDNAs, which are oriented as direct repeats and are separated by a drug selection marker. One of the GFP sequences is modified to contain the recognition site for the I-*Sce*I endonuclease. Consequently, a DSB will be introduced in the GFP sequence when I-*Sce*I is exogenously expressed (**Fig. 3A**). The HR event between the two GFP genes produces an intact *GFP* gene and expression of a functional GFP

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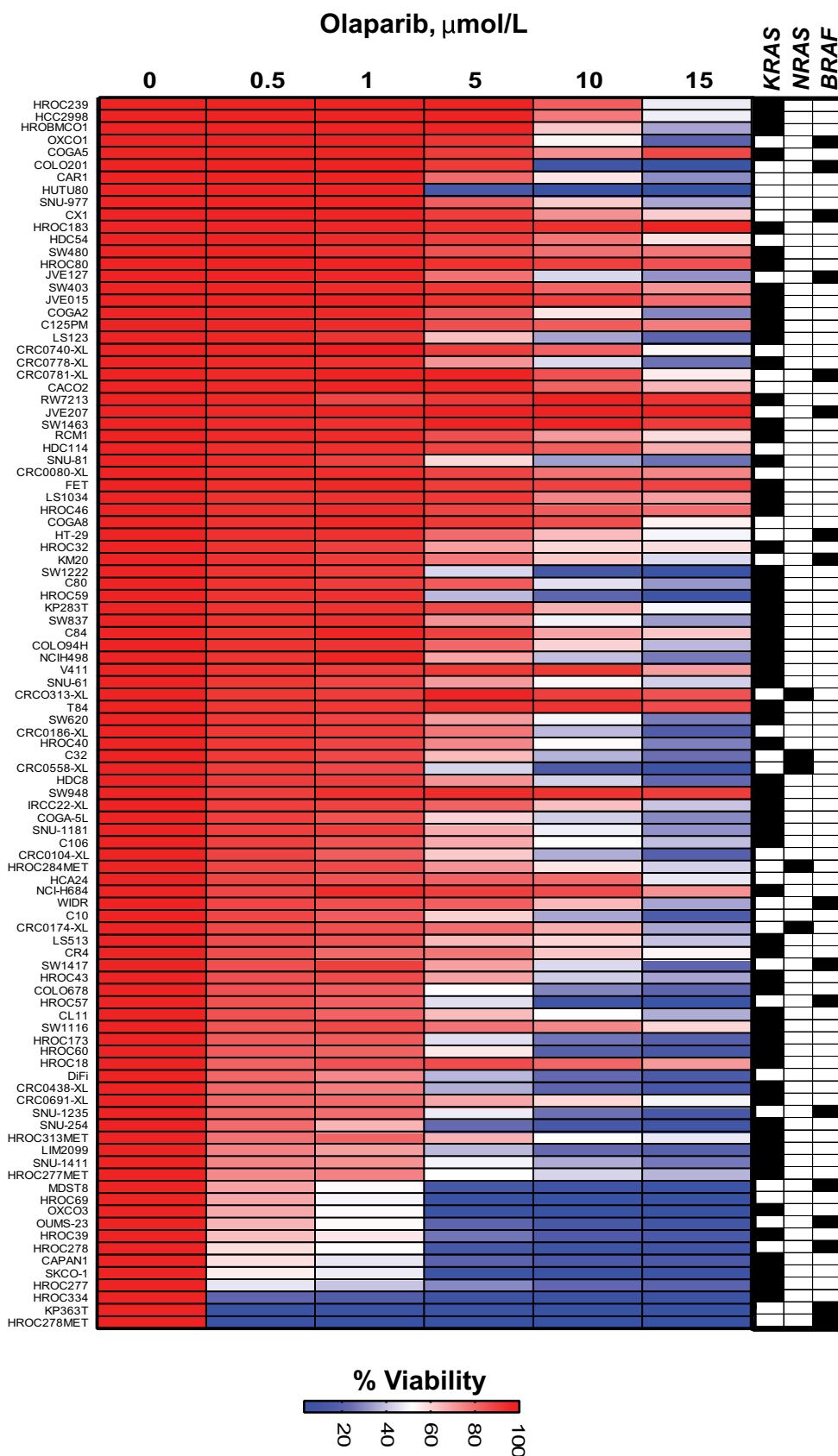
Figure 1.
A subset of colorectal cancer cells is sensitive to clinically relevant concentrations of the PARP inhibitor olaparib. Ninety-nine colorectal cancer cells carrying alterations in *RAS*, *BRAF*, or other genes conferring resistance to cetuximab (right; Supplementary Table S1) were tested for olaparib sensitivity by a long-term proliferation assay. DiFi, a colorectal cancer cell line sensitive to cetuximab, and CAPAN1, a BRCA2-deficient pancreatic cell line sensitive to olaparib, were included as controls. Each cell line was tested at least twice with technical duplicates. Heatmap was plotted with Graphpad Prism software.

Figure 2.
γ-H2AX and RAD51 profiles in olaparib-resistant and -sensitive cells upon ionizing radiation. **A,** Quantification of nuclear γ-H2AX foci (top) and RAD51 foci (bottom) in olaparib-resistant and -sensitive cells. Nuclei with five or more foci were scored as positive, and at least 500 nuclei were counted for each sample. A two-way ANOVA with a Tukey *post hoc* test was used to calculate the significance within the resistant (γH2AX: P = ns; RAD51: P < 0.0001) and sensitive (γH2AX: P = ns; RAD51: P = ns) subgroups comparing the treatment conditions (NT vs. 5 Gy and NT vs. 10 Gy). **B,** Immunofluorescence detection of DNA damage (γH2AX) and (**C**) a marker of HR (RAD51) in olaparib-resistant (COGA5) and -sensitive (KP363T) cells treated as indicated. Four hours after irradiation, cells were fixed and stained. Nuclei are stained with DAPI (blue) and anti-γ-H2AX antibody (red) or anti-RAD51 antibody (green). Scale bar = 50 μm. Representative images for two resistant and two sensitive cell lines are shown (see also Supplementary Fig. S3).
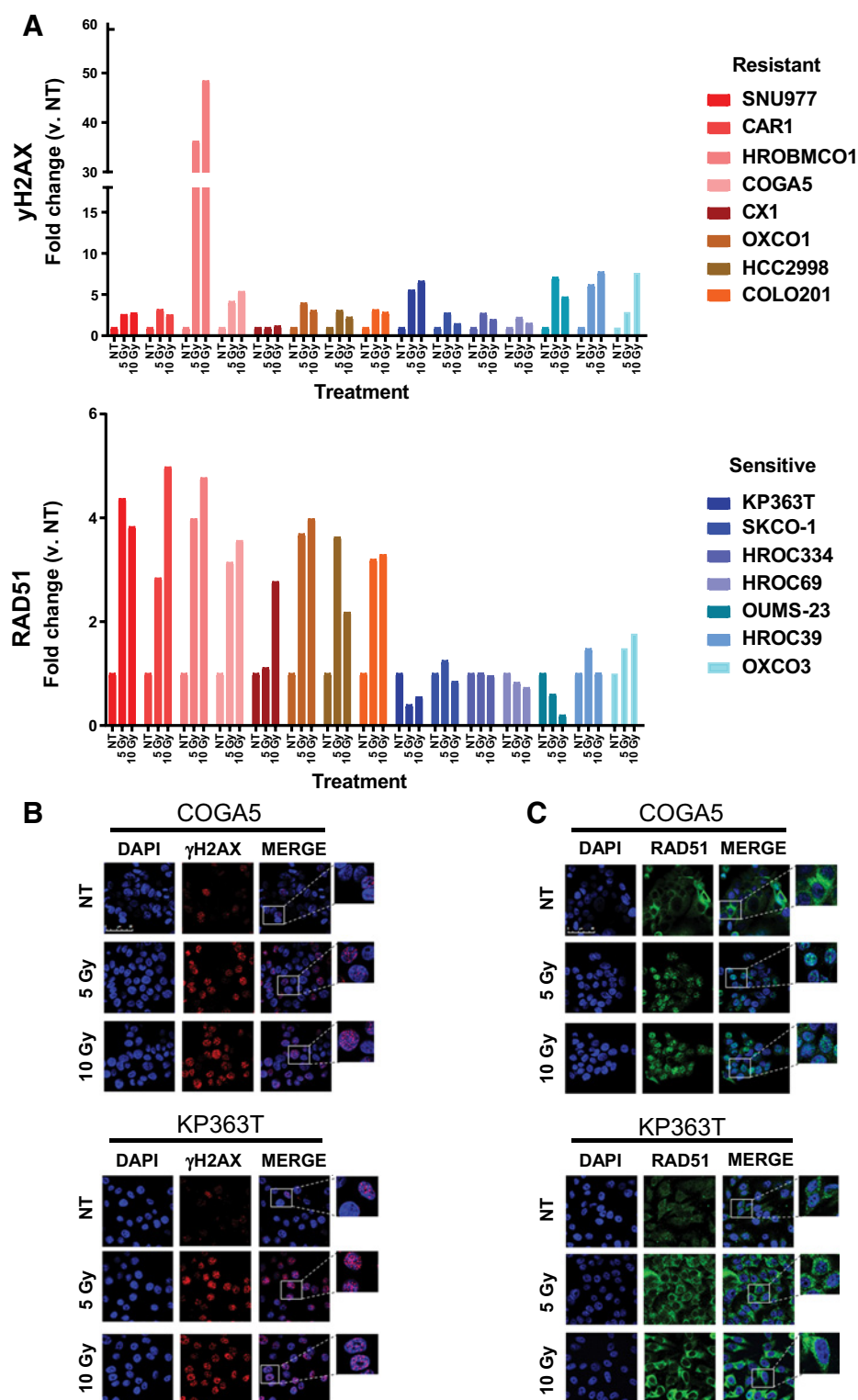
protein. The pDR-GFP plasmid was stably expressed in olaparib-sensitive and -resistant cells (selected on the basis of their transfection efficiency) and the green fluorescent signal was measured by flow cytometry. GFP protein expression was observed in the olaparib-resistant cells (WiDr and DiFi) following I-*SceI* transfection, indicating that these cells were able to effectively repair the damaged GFP DNA. In contrast, olaparib-sensitive cells such as HROC278MET, KP363T, and SKCO-1 were unable to repair the damaged DNA and therefore GFP expression was not detected following I-*SceI* transfection (**Fig. 3B**; Supplementary Fig. S4).

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A

B
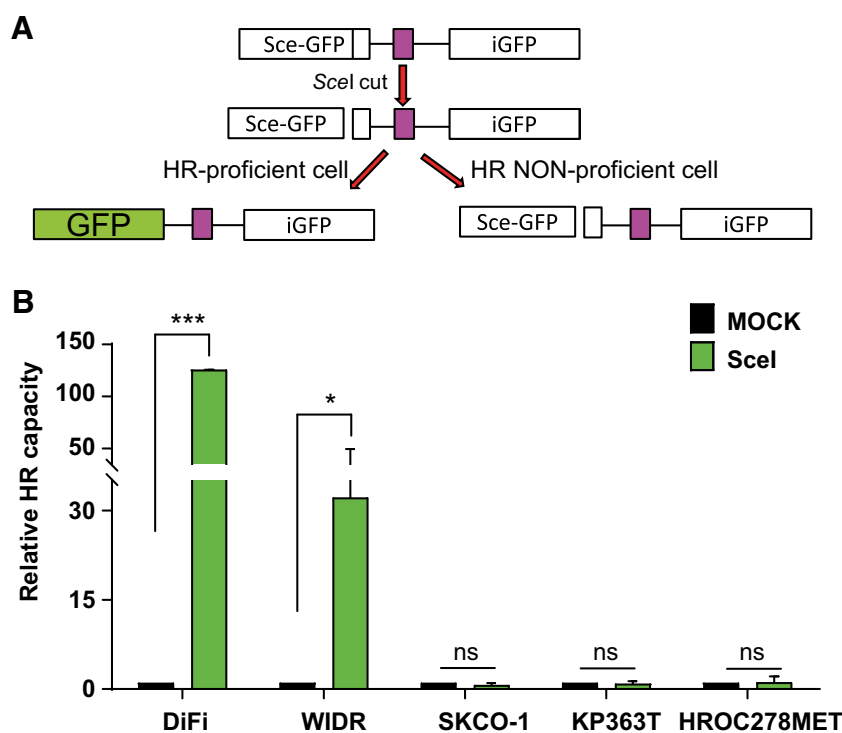
Figure 3.
HR assays in colorectal cancer cells sensitive and resistant to olaparib. **A,** Schematic representation of DSB repair HR reporter assays. The pDR-GFP reporter plasmid contains a SceGFP gene, which includes an I-SceI site and in-frame termination codons. An 812-bp internal GFP fragment (iGFP) can be used as a template to repair the DSB by HR-proficient cells that will generate a functional GFP gene whose green fluorescence can be detected by FACS. A puromycin selection gene is indicated in purple between the SceGFP and iGFP sequences. **B,** The indicated cells were initially transfected with the pDR-GFP plasmid. Next, stably expressing cells were transfected with the pCBASce-I to confer DNA damage and 50 to 60 hours after transfection cells were analyzed by flow cytometry. Quantification of HR capacity of each cell line relative to mock transfection (control) is reported in the bar graph. Results represent means ± SD of at least two independent experiments. Statistical significance: ns, not significant; *, $P < 0.05$; ***, $P < 0.001$ (Student t test).

Cross-sensitivity between olaparib and oxaliplatin in colorectal cancer cells

The results presented above highlight challenges in using genomic data to predict colorectal cancers that are susceptible to PARP inhibition. We noted that in ovarian cancers the clinical development of the PARP inhibitors has encountered similar difficulties in using genomic correlates to identify patients likely to respond (25, 54). In ovarian cancer, sensitivity to platinum-based therapy is associated with subsequent clinical benefit from PARP inhibition and response to platinum is used as a criterion to select patients likely to respond to PARP blockade (25–27). We therefore hypothesized that colorectal cancers sensitive to PARP blockade could also display cross sensitivity to oxaliplatin, a drug broadly used in combination regimens for treatment of bowel tumors (9). To test this possibility, we treated olaparib-sensitive and a subset of olaparib-resistant cells with a range of oxaliplatin concentrations (**Fig. 4A**, left and central). A significant correlation (Spearman $r = 0.63$, $P = 0.0005$,) between olaparib and oxaliplatin sensitivity was observed (**Fig. 4B**). To assess the specificity of this finding, we also tested sensitivity to 5-FU (another widely used chemotherapeutic agent) in the same cell models and found no correlation (**Fig. 4A**, right and **C**).

Olaparib and oxaliplatin sensitivity in patient-derived colorectal cancer organoids

Considering that in our experience molecular correlates are unable to unequivocally predict sensitivity or resistance to olaparib in colorectal cancer, we wondered whether patient-derived colorectal cancer organoids (PDO) could represent a functional platform to rapidly determine sensitivity to PARP blockade in this setting. We therefore analyzed five colorectal cancer organoids established either directly from tumor samples or from PDXs (Supplementary Table S4). A PDO derived from a *BRAF* mutant PDX (patient #1, case HROC278, **Fig. 5A–C**), from which we already derived olaparib-sensitive 2D cell lines (**Fig. 1**), displayed exquisite sensitivity to

olaparib. Two organoids—derived from a *NRAS* mutant colorectal cancer specimen (patient #2) or from an *HER2* amplified PDX (patient #3)—were also rather sensitive, although growth impairment could be appreciated only at higher concentrations of the PARP inhibitor. The remaining two models proved refractory across the entire range of olaparib concentrations (**Fig. 5A–C**). We next performed oxaliplatin tests on all five PDOs and found a striking cross sensitivity between olaparib and oxaliplatin (**Fig. 5A–C**).

To understand whether response to oxaliplatin treatment could be prospectively translated into prediction to olaparib response in patients, we collected clinical information on oxaliplatin-based treatment and response before tumor surgery and organoid generation in these five patients. Although patients #1 and #5 did not receive oxaliplatin in the previous round of therapy, patients #2, #3, and #4 were treated with oxaliplatin-containing regimens before surgery (Supplementary Table S4). Patients #2 and #3 achieved partial response (PR) to previous oxaliplatin-based therapy and the corresponding organoids are consistently sensitive to oxaliplatin. Intriguingly, patient #4 had derived clinical benefit from treatment with FOLFOX and panitumumab, but the organoids generated from his tumor were resistant to oxaliplatin. However, patient #4-derived organoids were highly responsive to panitumumab (Supplementary Fig. S5), suggesting that clinical benefit in this patient could be associated to EGFR blockade rather than to oxaliplatin.

Overall, these results indicate that PDOs, which can be rapidly derived from surgical or bioptic colorectal cancer samples, can be effectively exploited to determine olaparib sensitivity. Furthermore cross-sensitivity between oxaliplatin and olaparib was maintained in patient-derived models.

Sequential olaparib and oxaliplatin treatment in patient-derived colorectal cancer xenografts

We hypothesized that maintenance therapy with olaparib may be beneficial in colorectal cancers that have experienced tumor shrinkage
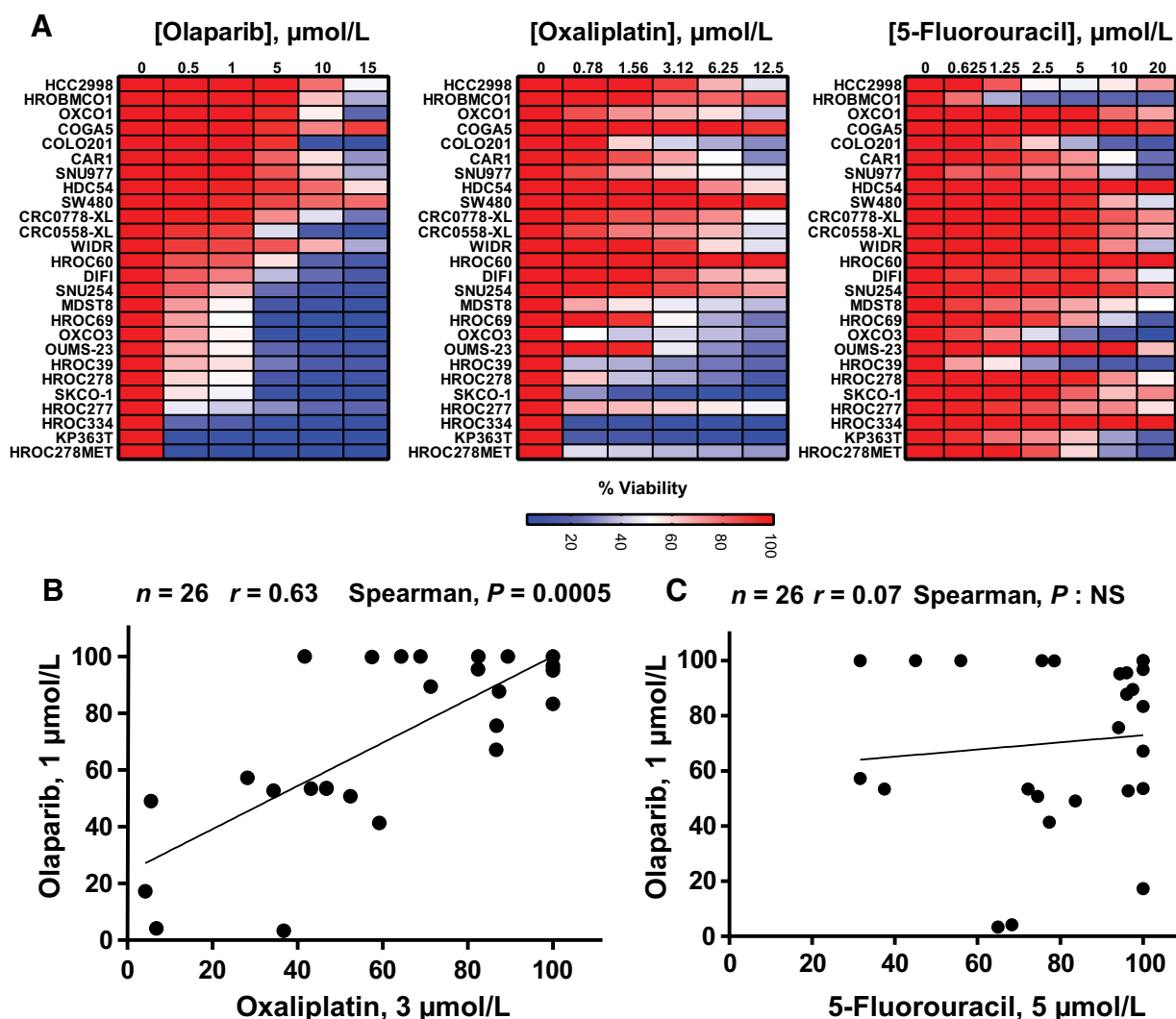
Figure 4.
Sensitivity to olaparib correlates with oxaliplatin activity in colorectal cancer cell lines. **A,** A subgroup of 26 cell lines initially screened with olaparib was tested with increasing concentrations of oxaliplatin (0–12.5 µmol/L) and 5-FU (0–20 µmol/L). **B,** Spearman analysis shows a significant correlation with oxaliplatin sensitivity ($P = 0.0005$), whereas no correlation was found with 5-FU response (**C**).

upon treatment with oxaliplatin-based regimens. We reasoned that *in vivo* treatment of PDXs might help to ascertain whether olaparib and oxaliplatin could effectively be used sequentially in colorectal cancers. This would have clinical relevance, for example when platinum toxicity becomes a limiting factor, a situation commonly experienced by patients with colorectal cancers. To test this, we selected case HROC278 (carrying BRAF V600E), from which we derived 2D lines and organoids that are sensitive to olaparib (**Figs. 1** and **5**). We had previously established two independent PDXs from this case, one from the primary tumor—HROC278—and the other from a metastatic lesion—HROC278MET. When these PDXs were tested, we observed prolonged tumor stabilization in olaparib-treated mice compared with vehicle-treated animals (**Fig. 6A** and **B**). We then hypothesized that maintenance therapy with PARP blockade after initial oxaliplatin response could be effective in HROC278 (**Fig. 6C**); indeed, treatment with olaparib delayed progression after initial oxaliplatin-mediated tumor shrinkage (**Fig. 6D**).

Discussion

In this work, we have assessed the antiproliferative activity of PARP inhibition in a collection of 99 colorectal cancer cell lines and found that up to 13% of them undergo growth arrest during 2-week exposure of clinically achievable levels of olaparib. We note that the most sensitive colorectal cancer lines were as responsive to olaparib as BRCA-deficient pancreatic or ovarian cancer lines (55). Although the primary screen was performed in two-dimensional tissue culture format, we also provide evidence that response to olaparib is maintained in clinically relevant models such as patient-derived organoid culture systems and xenograft models.

We elected to study microsatellite stable (MSS) cell lines which represent the patient population with the highest "unmet clinical need score"; because MSI colorectal cancers can be successfully treated with immunotherapy and, at least in the metastatic setting, are quite rare (56).
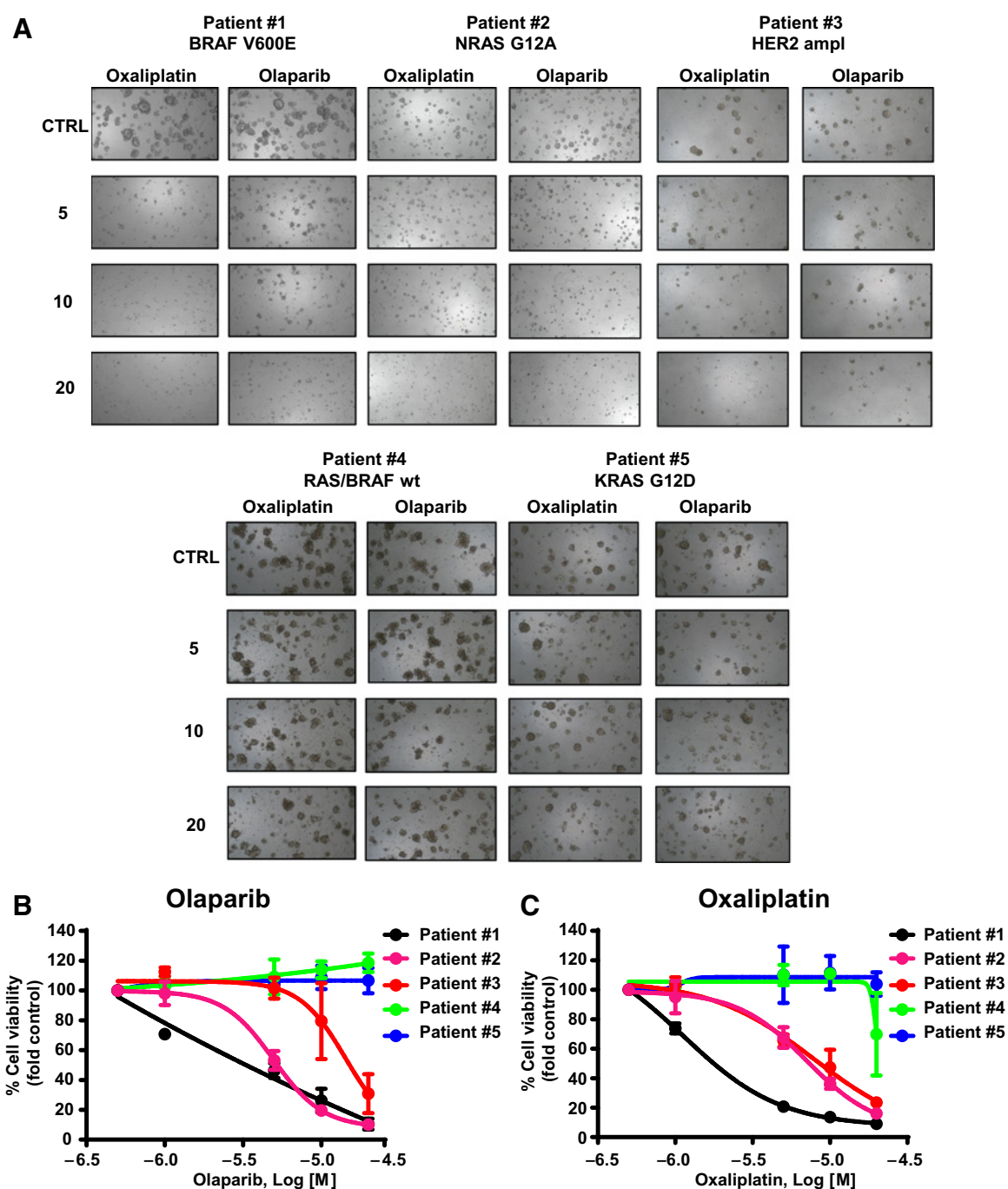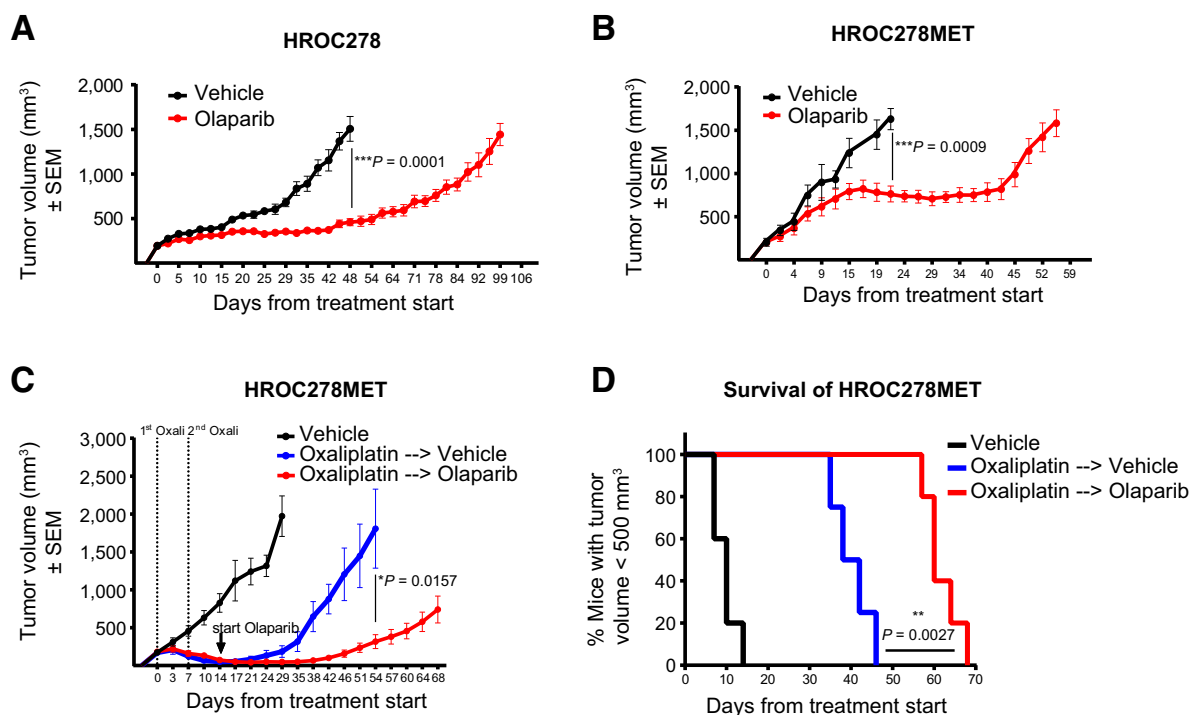
Figure 5.

Colorectal cancer patient–derived organoids shows cross-sensitivity to olaparib and oxaliplatin. **A,** Organoids derived from patients with colorectal cancer or PDXs were disaggregated at the single-cell level and plated on BME in a 96-well plate. Once culture was established, treatment with olaparib or oxaliplatin at the indicated concentrations (μmol/L) was performed for 18 days. At the end of the experiment, organoids were microphotographed in bright field, and representative pictures are shown. **B** and **C,** At the end of olaparib (**B**) or oxaliplatin (**C**) treatment, organoid viability was measured by CellTiter GLO assay. Results are average of at least two independent experiments with technical quadruplicates. Error bars represent SD.

We initially explored whether biomarkers predictive of clinical benefit from PARP inhibitors in other malignancies could be applied to identify colorectal cancer models responsive to olaparib. A clinically approved BRCA1 and BRCA2 diagnostic test did not identify mutations in either *BRCA1* or *BRCA2* previously associated with HR

deficiency and sensitivity to PARP inhibition. Exome sequencing was then employed to profile the status of HR genes. Although mutation profiles of DNA repair genes could not distinguish between olaparib-sensitive and -resistant lines, a relevant number of colorectal cancer lines carried one or more defects in genes involved in HR, leading to

Figure 6.
Assessment of sensitivity to olaparib as single agent or as maintenance therapy after oxaliplatin treatment in PDXs. **A** and **B,** PDXs obtained by two different lesions from the same patient, tumor (HROC278; **A**) and metastasis (HROC278MET; **B**), were tested for olaparib sensitivity (50 mg/kg), and treatment was started when tumors reached 200 mm^3 (day 0). **C,** The PDX model obtained from HROC278MET was challenged with olaparib as maintenance therapy after initial oxaliplatin treatment. Oxaliplatin (10 mg/kg) was administered intraperitoneally once a week on days indicated by the dashed lines, and olaparib treatment (50 mg/kg) was administered intraperitoneally starting after 7 days from second oxaliplatin treatment (black arrow). **D,** Progression-free survival of mice from experiment shown in **C**. A tumor volume >500 mm^3 was selected as an arbitrary endpoint. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed unpaired Student t test). The Kaplan–Meier method was used for survival analysis (D), and P value was calculated using the log-rank test (Mantel–Cox).

possible functional consequences. We acknowledge that we could not ascertain the germline or somatic nature of the identified variants likely to confer defective HR, because matched normal DNA was not available for most tumor cell lines. Importantly, the large amount of alterations in DNA damage response genes with unknown significance renders impracticable functional testing for individual mutations and this approach was not performed in this study.

Biallelic inactivation of *BRCA1* or *BRCA2* is associated with a pattern of genome-wide mutations known as signature 3, which reflects underlying deficient HR in breast cancer. Germline nonsense and frameshift variants in *PALB2*, as well as epigenetic silencing of *RAD51C* and *BRCA1* by promoter methylation, can also give rise to the same signature in breast cancer and has been proposed as a biomarker to select tumors that may benefit from PARP inhibition (48). The term "BRCAness" has been coined to describe tumors with features similar to those found in patients with germline *BRCA1* or *BRCA2* alterations, resulting in sensitivity to PARP inhibition and other DNA-damaging agents due to defective HR (15). Although we positively identified mutational profile signatures occurring in colorectal cancer such as numbers 6 and 10, only 2 of 13 olaparib-sensitive cells displayed signature number 3, which is correlated with HR defects and BRCAness (47, 49). In our experience, mutational signatures could not discriminate between olaparib-sensitive and -resistant colorectal cancers.

In summary, our data suggest that genomic features associated with BRCAness or HR repair diagnostic assays (at least with current computational methods) do not entirely capture colorectal cancer

tumors susceptible to PARP inhibition. On the contrary, we found that DNA repair functional tests are effective in discriminating sensitive and resistant colorectal cancer lines. Our results are concordant with previous studies in breast cancer organoid and PDX lines indicating that RAD51 nuclear foci are a surrogate marker of HR repair functionality (57, 58). Future studies should test the feasibility of detecting RAD51 in clinical colorectal cancer specimens.

We also found that sensitivity to oxaliplatin as determined in colorectal cancer models correlates with response to PARP blockade. In this regard, clinical development of the PARP inhibitors niraparib and rucaparib has been focused on platinum-sensitive ovarian cancer, thereby preferring clinical selection to molecular selection criteria. Sensitivity to platinum-based chemotherapy in ovarian cancer is associated with subsequent clinical benefit from PARP inhibition (21, 25–27). Intriguingly, most of the colorectal cancer lines responsive to olaparib show cross-sensitivity to oxaliplatin, suggesting that oxaliplatin efficacy could be exploited to define "clinical BRCAness."

Importantly, we report that PDOs can be used to predict sensitivity to PARPi and that previous response to oxaliplatin-based regimens might predict for response to olaparib in patients with colorectal cancer. It is therefore tempting to conclude that rapid establishment of organoids from clinical samples could in the future be effectively used in precision medicine programs aimed at selecting patients with colorectal cancer likely to respond to PARP blockade.

Previous evidences in small datasets suggest that oxaliplatin could be efficacious in gastrointestinal tumors with HR defects

such as pancreatic cancers (23, 59). Two recent case reports indicate that patients with *BRCA1/2* mutant rectal tumors achieved a complete pathologic response upon treatment with oxaliplatin-containing neoadjuvant chemotherapy (11, 60). A phase II study of single agent olaparib in chemo-refractory mCRC was terminated prematurely due to lack of objective responses (30). A phase I study of olaparib in combination with irinotecan indicated increased toxicity and lack of clinical efficacy (61). However, these trials were conducted in a heavily pretreated population of mixed MSS/MSI mCRC cases, without providing information about response to prior oxaliplatin treatment.

Our findings instead suggest that in mCRC PARP inhibition could be tested as maintenance therapy in the subset of patients characterized by underlying "BRCAness" features and who achieved significant tumor shrinkage after FOLFOX-based induction first-line chemotherapy.

Because combinations of PARP inhibitors with oxaliplatin or irinotecan may be toxic (61), further opportunities for exploiting PARP inhibition in the clinic may come from combination with immune checkpoint inhibitors (https://clinicaltrials.gov ID: NCT03851614; ref. 62).

Overall, the results presented in our work can be easily translated into clinical trials testing the efficacy of PARP inhibitors in patients with mCRC who carry HR-deficient tumors and have experienced profound tumor shrinkage upon induction first-line FOLFOX-chemotherapy. Contrary to the trials previously conducted in all comers, this therapeutic strategy is expected to improve progression-free survival and curb those quality-of-life-impairing side effects, such as neurotoxicity, associated to a prolonged oxaliplatin exposure.

Disclosure of Potential Conflicts of Interest

M. Pagani holds ownership interest (including patents) in CheckmAb s.r.l. A. Amatu is an employee/paid consultant for Roche and Bayer. A. Sartore-Bianchi is an advisory board member/unpaid consultant for Amgen, Bayer, and Sanofi. S. Siena is an employee/paid consultant for Amgen, Bayer, Brisol-Myers Squibb, CheckMab, Clovis, Daiichi Sankyo, Incyte, Merck, Novartis, Roche-Genentech, and Seattle Genetics. A. Bardelli reports receiving commercial research grants from Neophore; is an advisory board member/unpaid consultant for Roche, Illumina, Guardant, and Third Rock; holds ownership interest (including patents) in Neophore and Phoremost; and is an advisory board member/unpaid consultant for Horizon Discovery, Biocartis, and Neophore. No potential conflicts of interest were disclosed by the other authors.

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A Subset of Colorectal Cancers with Cross-Sensitivity to Olaparib and Oxaliplatin

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