Exposure to escalating olaparib does not induce acquired resistance to PARPi and to other chemotherapeutic compounds in ovarian cancer cell lines

ANDRÉ FEDIER[1], NADIA MAGGI[1], ALESSANDRA TOZZI[2], MURIEL DISLER[1], RICARDO COELHO[1], FRANCIS JACOB[1,3] and VIOLA HEINZELMANN-SCHWARZ[1,2]

[1]Ovarian Cancer Research Program, Department of Biomedicine, University Hospital Basel, University of Basel, 4031 Basel; [2]Hospital for Women, Department of Gynecology and Gynecological Oncology, Women's Hospital, University Hospital Basel, University of Basel, 4021 Basel; [3]Glyco-Oncology, Ovarian Cancer Research, Department of Biomedicine, University Hospital Basel, University of Basel, 4031 Basel, Switzerland

Received September 20, 2021; Accepted May 17, 2022

DOI: 10.3892/ijo.2022.5379

Abstract. Poly (ADP-ribose) polymerase (PARP)-inhibitors (PARPi) such as olaparib and niraparib are currently used as a treatment option for *BRCA*-deficient tumors and also show efficacy in platinum-sensitive tumors. However, resistance to PARPi occurs in numerous patients and in particular acquired PARPi resistance presents a major obstacle in the treatment of these tumors. In the present study, it was investigated whether stepwise exposure of ovarian cancer cells to escalating concentrations of olaparib produced subcells with acquired resistance to PARPi and/or acquired cross-resistance to platinum compounds, paclitaxel, and doxorubicin. To this aim, the sensitivity of fourteen ovarian cancer cell lines, including nine with *TP53*-mutations and five carrying *BRCA*-mutations, to olaparib and niraparib was determined and a subset of seven cell lines was selected to investigate the potential of olaparib to produce resistance. It was identified that escalating olaparib did neither produce subcells with acquired PARPi-resistance nor did it produce acquired cross-resistance to platinum compounds, doxorubicin, and paclitaxel. This finding was independent of the cells' *TP53* and *BRCA* mutation status. CRISPR-*Cas9* mediated deletion of *PARP1* did not affect sensitivity to PARPi, platinum compounds, doxorubicin, and paclitaxel. In addition, olaparib sensitivity correlated with niraparib sensitivity, but *BRCA*-mutated cells were not more sensitive to PARPi. Moreover, PARPi sensitivity associated with cross-sensitivity not only to platinum compounds but also to anthracylines, paclitaxel, and inhibitors of histone deacetylases. These *in vitro* data indicated that olaparib exposure is unlikely to produce an acquired resistance phenotype and that PARPi-sensitive ovarian cancer cells are also cross-sensitive to non-platinum and even to compounds not directly interacting with the DNA.

Correspondence to: Dr André Fedier, Ovarian Cancer Research Program, Department of Biomedicine, University Hospital Basel, University of Basel, Hebelstrasse 20, 4031 Basel, Switzerland
E-mail: andre.fedier@usb.ch

Key words: acquired resistance, ovarian cancer, poly (ADP-ribose) polymerase-inhibitor, platinum, paclitaxel, anthracyclines, multidrug resistance, chemo-sensitivity, cross-resistance

Introduction

Chemo- and immunotherapy resistance is, besides incomplete cytoreductive surgery and tumor metastasis, a major cause for recurrence and cancer therapy failure. Therapy resistance can be either intrinsic where tumors are not sensitive to therapy due to existing resistance-causing factors or acquired where initially sensitive tumors become resistant during therapy. Major causes for drug resistance have been extensively studied in the past: elevated expression of drug efflux transporters, increased or decreased DNA-damage repair, altered drug metabolism and detoxification, mutated drug targets, altered survival/death signaling, hypoxia, and presence of cancer stem cells (1,2).

Poly (ADP-ribose) polymerase (PARP)-inhibitors (PARPi) such as olaparib and niraparib are successfully used as a treatment of platinum-sensitive Breast Cancer Susceptibility Gene (*BRCA)*-deficient tumors of the breast and the ovaries. They are also effective in tumors with a '*BRCA*ness'-phenotype, that is tumors with no mutations in *BRCA* genes but with loss-of-function mutations in genes encoding other key players in the homologous recombination repair (HRR) pathway. *BRCA1* and *BRCA2* are crucial for the HRR process. PARPi are small-molecule inhibitors of PARP enzymes 1, 2, and 3, which are involved in detection and repair of single-strand breaks (SSB). PARPi block PARP catalytic activity or 'trap' PARP molecules onto the DNA, resulting in SSB persistence, replication fork stalling, and accumulation of DNA double-strand breaks (DSB), eventually leading to a 'synthetic lethality' phenotype in an HRR-deficient (HRD) background (3-9). However, emerging evidence indicates that PARPi are even effective in cells with functional HRR (10).

Although *BRCA*-mutations are a favorable predictor for PARPi sensitivity, a significant number of *BRCA*-mutated cancer nevertheless fail to respond to PARPi (11-14), either due to intrinsic resistance or resistance acquisition during the treatment and possibly enforced by the 'mutator phenotype' of HRD cells. Mechanisms of PARPi resistance include the restoration of HRR through reversion mutations in *BRCA* and *RAD51*, through *BRCA1* promotor alterations, or through reconstitution of end-resection; the occurrence of alterations of PARP1 that abolish PARPi-trapping; the stabilization of the replication fork via depletion of chromatin remodelers; and the increased PARPi efflux via overexpression of multidrug resistance (MDR) pumps (15-17).

Reversion mutations (insertions or deletions) 'stamp out' pathogenic *BRCA* or *RAD51* mutations and thus re-establish the reading frame, remove the deleterious mutation, or cause a synonymous mutation, restoring functional *BRCA* and RAD51 (18-20). Restoration of homologous recombination (HR) with coinciding BRCA protein re-expression due to de-methylation of *BRCA1* was demonstrated in patient-derived xenograft (PDX) models of PARPi-resistant breast cancer (21) as well as in patients with ovarian cancer (22). HR restoration can also occur by loss of the end-resection repressor 53BP1 (23) or depletion of the shielding complex component REV7 (24).

The occurrence of mutations in the *PARP1* gene is the most obvious cause for abolished PARPi-trapping: These mutations may result in a PARP protein unable to become trapped in response to PARPi. This has been found in an ovarian cancer patient with olaparib *de novo* resistance: she had a PARP1 mutation affecting a region critical for the cross-talk between the DNA-binding and catalytic domains (25). Phosphorylation of PARP1 was also found to reduce binding by PARPi, increase PARP1 enzymatic activity, and thus confers resistance to PARPi (26).

The dysregulation or collapse of replication forks, where for instance a variety of fork- and chromatin-remodeling proteins promote MRE11-dependent nascent DNA strand degradation at stalled replication forks, is one important feature of PARPi cytotoxicity. Depletion of these remodelers prevented strand degradation by MRE11, leading to the stabilization of the replication fork, and resistance to olaparib in *BRCA1/2* deficient cells (27,28).

MDR1 overexpression is a common feature for MDR and the negative impact of MDR1 overexpression on olaparib-sensitivity was shown in a mouse model (29) and also in breast and ovarian cancer cases where PARPi-resistance could be attributed to fusions and rearrangements of genes located near *ABCB1* (30).

The issue whether PARPi exposure can generate an acquired resistance phenotype in cancer cells has only been poorly addressed, with controversial results been reported. Two studies reported development of acquired PARPi resistance in ovarian cancer cells by olaparib (31) and in breast cancer cells by niraparib (32) whereas another study reported that niraparib failed to induce mutations responsible for treatment resistance (33).

Thus, it remains unclear whether PARPi-imposed resistance acquisition is a common or a rather rare phenomenon, whether it is cell line-dependent, whether it depends on the mutational profile of genes implicated in DNA damage pathways (e.g. *BRCA*, *TP53*) or on the genetic background in general, whether it occurs preferably in cells that are intrinsically PARPi-sensitive, and whether it is a permanent or rather a transient (resistance phenotype is lost after removal of the selection pressure) phenomenon. In addition, it is unclear whether cells with an acquired PARPi resistance are cross-resistant to PARPi-unrelated compounds such as platinum salts, anthracyclines and even compounds not directly interacting with the DNA like Paclitaxel.

To address these topics, the intrinsic sensitivity of a panel of fourteen ovarian cancer cell lines to olaparib and niraparib as well as to PARPi-unrelated compounds was first determined, and then a subpanel of seven cell lines (six with and one without *TP53*-mutations, and two with and five without *BRCA*-mutations) was selected to investigate whether exposure to escalating olaparib concentrations generates subcells with acquired resistance to PARPi and/or PARPi-unrelated compounds.

Materials and methods

Cell culture and drugs. The following fourteen parental cell lines were used: A2780, BG1, CaOV3, COV362, ES-2, IGROV1, Kuramochi, OVCAR3, OVCAR4, OVCAR5, OVCAR8, OVSAHO, SKOV3, and TYK-nu. Nine of them are *TP53*-mutated and five *BRCA*-mutated (Fig. 1). Cisplatin-resistant A2780/CP were obtained from parental A2780 cells by stepwise exposure to increasing cisplatin concentrations (35). The cisplatin-resistant TYK-nu(R) cells were developed from parental TYK-nu cells by stepwise exposure to cisplatin and obtained from JCRB Japan Cell Bank (36). Paclitaxel-resistant IGROV1-PXL were generated from parental IGROV1 cells through stepwise exposure to escalating concentrations of paclitaxel in our lab (37). All cell lines were cultured in RPMI-1640 (cat. no. R8758) supplemented with 10% fetal bovine serum (FBS; both from Sigma-Aldrich; Merck KGaA), penicillin/streptomycin (100 U/ml/100 μg/ml; Sigma-Aldrich; Merck KGaA) at 37˚C in a 95% humidified atmosphere containing 5% CO_2. All cell lines are STR-profiled and routinely tested for mycoplasma infection.

Chemotherapeutic drugs were obtained from various suppliers: Gloucester Pharmaceuticals Inc. (romidepsin); Labatec (carboplatin); MedChemExpress (OTS167); Sigma-Aldrich; Merck KGaA [cisplatin, doxorubicin, epirubicin, paclitaxel and suberoylanilide hydroxamine (SAHA)]. They were dissolved in DMSO (niraparib, olaparib and SAHA), methanol (paclitaxel), or water (carboplatin, cisplatin, doxorubicin, epirubicin, OTS167 and romidepsin) and stored as aliquots at -20˚C.

Drug sensitivity and cell proliferation rate. Drug sensitivity was determined by the MTT-assay and the colony formation assay (CFA). For the MTT-assay, cells (5,000-7,000 in 200 ml medium: density depends on the cell line) were seeded into 96-well plates and treated with each drug for 72 h: carboplatin (range: 3-500 μM), cisplatin (0.5-50 μM), doxorubicin (3-3,000 nM), epirubicin (0.1-10 μM), niraparib (0.5-100 μM), olaparib (3-1,000 μM), OTS167 (10-1,000 nM), paclitaxel (0.5-300 nM), romidepsin (0.1-100 nM), SAHA (0.5-300 μM).

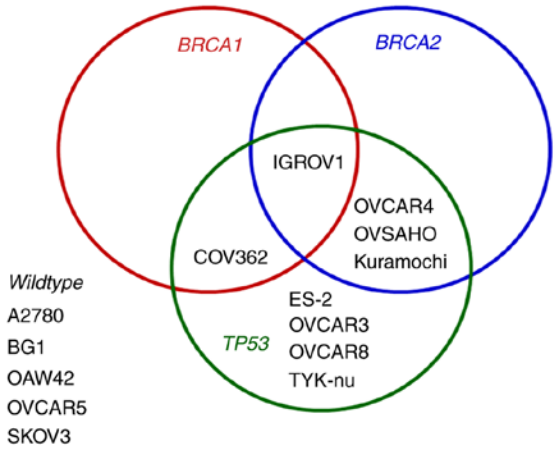

Figure 1. Schematic presentation of the *TP53*, *BRCA1* and *BRCA2* mutational status of the fourteen cell lines under study. Sources: Domcke *et al*., 2013 (ref. 34) and 'Cell Model Passport' (https://cellmodelpassports.sanger.ac.uk). OVSAHO cells have a homozygous deletion of *BRCA2*. Cell lines in bold were selected for the resistance acquisition protocol.

Then MTT-dye (cat. no. M2128; Sigma-Aldrich; Merck KGaA; final concentration: 0.5 mg/ml) was added for 3 h, followed by removal of the medium and dissolution of the purple formazan crystals with DMSO. The optical density (OD; absorbance at 540 nm) was measured using the SynergyH1 Hybrid Reader (BioTek Instruments, Inc.). Data (mean ± SD of at least four independent experiments performed in quadruplets) are presented as the relative proliferation as a function of time after seeding. IC_{50}-values were calculated by linear extrapolation. The ratio of the IC_{50}-values of the matched subcells and the parental cells was calculated. Subcells were considered resistant if the ratio was ≥2.0 or hypersensitive if ≤0.5.

For the CFA, 1,000 cells in 2-ml culture medium were seeded into 12-well plates and exposed to olaparib on the next day for 8-10 days: 0.1, 0.2, 0.5, 1 μM olaparib for A2780 cells; 4, 8, 16, 32 μM for IGROV1; 0.3, 1, 3, 10 μM for OVCAR3; 1, 2, 5, 10 μM for OVCAR8. Then the medium was removed, the colonies were fixed at room temperature for 30 min and stained with 0.05% crystal violet (Sigma-Aldrich; Merck KGaA) in 4% formalin (Formafix AG), the plates were then rinsed 3-4 times with water and dried and images of the plates were captured (Fusion FX7 Edge Imaging System; Witek AG).

Cell proliferation rate was calculated from cell counts at the seeding day and the harvesting day by the following formula for exponential growth: Td = T1 x log(2)/log(N1/N0): where N0 is the number of cells seeded at time T0, N1 the number of cells harvested after time T1 and Td the doubling-time (proliferation rate).

Western blotting. Western blot analysis was used to assess the expression of MDR1. Cell lysates were obtained from subconfluent cultures at the time of harvest. Cells were lysed with RIPA buffer (cat no. 9806; Cell Signaling Technology Europe). Protein concentration was determined by the BCA Protein Assay (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.). A total of 20 μg of protein were loaded and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE), followed by blotting onto a polyvinylidene difluoride (PVDF) membrane (cat. no. 162-0177; BioRad Laboratories, Inc.) according to standard protocols. Membranes were blocked at room temperature for 60 min in TBST/milk [Tris-buffered saline with 0.1% Tween® 20 (Sigma-Aldrich; Merck KGaA)] and containing 3% (w/v) fat-free milk powder purchased from a local grocer). Cas9, EGFP, GAPDH, MDR1, PARP1 and tubulin were detected with specific primary antibodies: mouse anti-Cas9 (cat. no. 14697; Cell Signaling Technology, Inc.), mouse anti-EGFP (cat. no. sc-9996; Santa Cruz Biotechnology, Inc.), mouse anti-GAPDH (cat. no. sc-47724; Santa Cruz Biotechnology, Inc.), mouse anti-MDR1 (cat. no. sc-13131; Santa Cruz Biotechnology, Inc.), rabbit anti-PARP1 (cat. no. 9542; Cell Signaling Technology, Inc.) and rabbit anti-tubulin antibody (cat. no. 2148; Cell Signaling Technology, Inc.). All primary antibodies were diluted 1:1,000 in TBST/milk, except MDR1 which was diluted 1:500). Following the primary incubation (at 4˚C for overnight), the membrane was incubated at room temperature for 3 h with the matched secondary antibodyeither horseradish peroxidase (HRP)-conjugated anti-mouse (cat. no. 7076) or HRPO-conjugated anti-rabbit (cat. no. 7074; both from Cell Signaling Technology, Inc.) antibody (both diluted 1:2,000 in TBST/milk) Complexes were visualized by enhanced chemiluminescence (Dura West; Pierce; Thermo Fisher Scientific, Inc.) and autoradiography (Fusion FX7 Edge Imaging System, Witek AG).

Generation of cell lines with acquired resistance. The following protocol was used to generate cells with acquired resistance to olaparib. It is based on the selection principle of clonal growth by repeated exposure of cells to stepwise escalating drug concentrations, assuming that cells acquire new features in an irreversible fashion by chronic drug exposure, as previously described (38).

The seven cell lines subjected to this protocol were selected according the following criteria. They exhibited different status for *TP53*, *BRCA1* and *BRCA2* (Fig. 1) and displayed different intrinsic olaparib sensitivity, ranging from relatively sensitive to relatively resistant (Fig. 2A): A2780, ES-2, IGROV1, OVCAR3, OVCAR4, OVCAR8, and TYK-nu). Briefly, 50,000 cells were seeded into six-well plates and exposed to olaparib for 48 h, followed by replacement of the olaparib-containing medium by olaparib-free medium in order to allow viable cells to recover and expand to confluency. After recovery, cells were re-seeded and exposed to a higher concentration of olaparib for 48 h, again followed by replacement of the olaparib-containing medium and recovery and expansion of viable cells. These cycles of exposure with escalating olaparib concentrations were repeated until no viable cells were left anymore in the last step of the protocol. This protocol and in particular the 48 h-exposure was selected because it was able to produce histone deacetylase inhibitor resistance acquisition in our previous studies (38-40). However, it is considered worthwhile trying different experimental protocols and conditions to induce PARPi resistance in future studies. The following matched subcells were obtained: A2780-OLA, ES-2-OLA, IGROV1-OLA, OVCAR3-OLA, OVCAR4-OLA, OVCAR8-OLA, TYK-nu-OLA. Protocol details are summarized in Table I (the start and end concentration of olaparib,

the number of cycles and the total olaparib escalation for each cell line). For each cell line the protocol was performed twice.

In order to monitor potential olaparib resistance acquisition, matched subcells and parental cells were subjected to MTT assays after each cycle. Resistance was defined if the ratio of the IC_{50}-values of the subcells and the parental cells is ≥2.0.

Generation of CRISPR-Cas9-mediated PARP1-knockout ovarian cancer cell lines. For molecular cloning, single guide RNAs (sgRNA) targeting protein-coding genomic DNA sequences of *PARP1* gene, exon 1 sg1_PARP1_5'-CGAGTC GAGTACGCCAAGAG-3', exon 2 sg2_PARP1 5'-TGGGTT CTCTGAGCTTCGGT-3', and exon 1 sg3_PARP1_5'-GCA TCCCCA AGGACTCGCTC-3', were designed using Benchling (Biology Software, 2021, retrieved from https://benchling.com). Single strand oligonucleotides were purchased from Sigma-Aldrich; Merck KGaA and cloned into LRG2.1 (cat. no. 108098; Addgene, Inc.) plasmid using the *BsmBI* endonuclease restriction site. Annealed oligonucleotides were ligated into the desired plasmid using the T4-DNA ligase (Promega Corporation) for subsequent expression of the sgRNA together with EGFP fluorescent protein. Ligations were transformed into Stbl3 *E. coli* following ampicillin selection using ZR Plasmid Miniprep-Classic plasmid purification (Zymo Research Corp.) and Sanger DNA sequencing (Microsynth AG) was used to confirm insertion of respective sgRNA using the human U6 primer (5'-GAGGGCCTATTT CCCATGATT-3').

Constitutively *Cas9*$^+$ expressing ovarian cancer cell lines were generated by lentiviral transduction and subsequent puromycin selection. In brief, 293T cells (kindly provided by Dr Neutzner, Department of Biomedicine, University Hospital Basel) were seeded in a T75 flask at 50% confluency one day before transfection for preparation of lentiviral particles. A total of 4 μg of LRG2.1 (cat. no. 108098) or pLenti-*Cas9*-P2A-Puro (ca. no. 110837), 2 μg of pMD2.G (cat. no. 12259) and 2 μg of pCMVR8.74 (cat. no. 22036; all from Addgene, Inc.) were co-transfected using 24 μl of jetPEI reagent in 1 ml of 150 mM NaCl solution (Polyplus-transfection; Chemie Brunschwig AG). Growth medium was changed 24 h after transfection. Supernatant containing-lentivirus particles was collected 48 h later and filtered with a 0.45-μm PVDF filter (Sartorius AG), aliquoted in cryotubes and stored at -80˚C until further use. OVCAR3, OVCAR5, and OVCAR8 cells were transduced with 1 ml of supernatant containing pLenti-Cas9-P2A-Puro lentivirus particles and further selected with 1-3 μg/ml puromycin for one week. Selected *Cas9*$^+$ cell lines were kept in media containing 1 μg/ml puromycin. Cells lines stably expressing Cas9 protein were then lentiviral-transduced either with sgRNAs targeting the *AAVS1 loci* (mock) (41); or *PARP1* followed by sorting enrichment of EGFP$^+$ cells using the BD FACSAria Cell Sorter (BD Biosciences).

To analyze and confirm CRISPR-mediated mutagenesis, genomic DNA of *Cas9*$^+$ non-gRNA transduced (control) or transduced cells with sgRNAs targeting *AAVS1* (mock) sg1_ AAVS1_5'-ACTGTTGACGGCGGCGATGT-3', sg2_AAVS1_ 5'-GCTGATACCGTCGGCGTTGG-3'; or *PARP1* sg1_ PARP1_5'-CGAGTCGAGTACGCCAAGAG-3, sg2_PARP1 5'-TGGGTTCTCTGAGCTTCGGT-3', sg3_PARP1_5'-GCA TCCCCA AGGACTCGCTC-3' was extracted using the DNeasy Blood & tissue Kit (cat. no. 69504 Qiagen) 3 and 6 days after transduction. The genomic *locus* targeted by *PARP1* was amplified using the forward 5'-GGGGGAGGG GTTGGGGGTAAAA-3' and reverse 5'-GCCTTCAAG CCCACCACCTCAC-3' primers. PCRs were performed using 2xGoTAq green Master Mix (Promega Corporation), 200 nM of each primer and 100 ng of genomic DNA. PCR conditions were as follows: Initial DNA denaturation at 94˚C for 5 min followed by 32 cycles of 95˚C for 20 sec, 62˚C for 15 sec and 72˚C for 1 min and 30 sec with a final extension at 72˚C for 5 min. Amplicons were visualized on 1% agarose gel and purified by Wizard SV gel and PCR Clean/up System (Promega Corporation). Amplicons were analyzed using the Tracking of Indels by Decomposition (TIDE) assay (42).

Statistical analysis. For all comparisons, the mean ± SD values were calculated and statistical analysis was performed using the paired, two-tailed Student's t-test (Microsoft Excel, version 2016). $P<0.05$ was considered to indicate a statistically significant difference. For correlation analyses, the Spearman's rank correlation was calculated (Microsoft Excel).

Results

PARPi-sensitive ovarian cancer cells are not only sensitive to platinum but also to other chemotherapeutic compounds. At first the sensitivity of fourteen cell lines (*TP53*-mutated, n=9; *BRCA*-mutated, n=5) (Fig. 1), to olaparib and niraparib was determined. The results demonstrated that these cell lines display a wide spectrum of olaparib and niraparib sensitivity, with ES-2 and BG1 as the most sensitive and COV362 and OVSAHO as the least sensitive cells to olaparib (Fig. 2A), and with BG1 and TYK-nu the most sensitive and OVCAR8 and COV362 the least sensitive cells to niraparib (Fig. 2B). Spearman's rank correlation analysis (Fig. 2C) showed that olaparib-sensitive cells were commonly also niraparib-sensitive (rs=0.582). They also indicated that among the fourteen cell lines, those with *BRCA*-mutations (COV362, Kuramochi, OVCAR4, IGROV1) tended to be less sensitive to both PARPi than those without *BRCA*-mutations (Fig. 2A-C). No correlation was found between the proliferation rate of ovarian cancer cells and their sensitivity to olaparib or niraparib (Fig. 2D).

Then it was determined whether PARPi-sensitive cells were, in addition to expectedly being sensitive to platinum compounds, also sensitive to other classes of chemotherapeutic compounds, such as representatives of the classes of anthracyclines, taxanes, histone deacetylase (HDAC)-inhibitors (HDACi), and maternal embryonic leucine zipper kinase (MELK)-inhibitor. The latter three are not known to interact with the DNA. MELK expression has recently been shown to correlate with poor outcome in ovarian cancer and its inhibition by OTS167 abrogates proliferation and viability of ovarian cancer cells (43). HDACi act epigenetically, that is without directly interacting with the DNA, and induce acetylation of histones and non-histone proteins and thus control gene transcription, protein function, proliferation and apoptosis (44). They have been shown to inhibit the growth and

Table I. Generation of subcells by escalating olaparib concentrations.

Subcell line	Concentration start (μM)	Concentration end (μM)	Number of cycles	Escalation (fold)
A2780-OLA	500	1080	2	2.16
ES-2-OLA	30	1800	5	60
IGROV1-OLA	200	1400	6	7
OVCAR3-OLA	250	800	8	3.3
OVCAR4-OLA	160	1200	4	7.5
OVCAR8-OLA	200	1200	4	6
TYK-nu-OLA	125	600	3	4.8

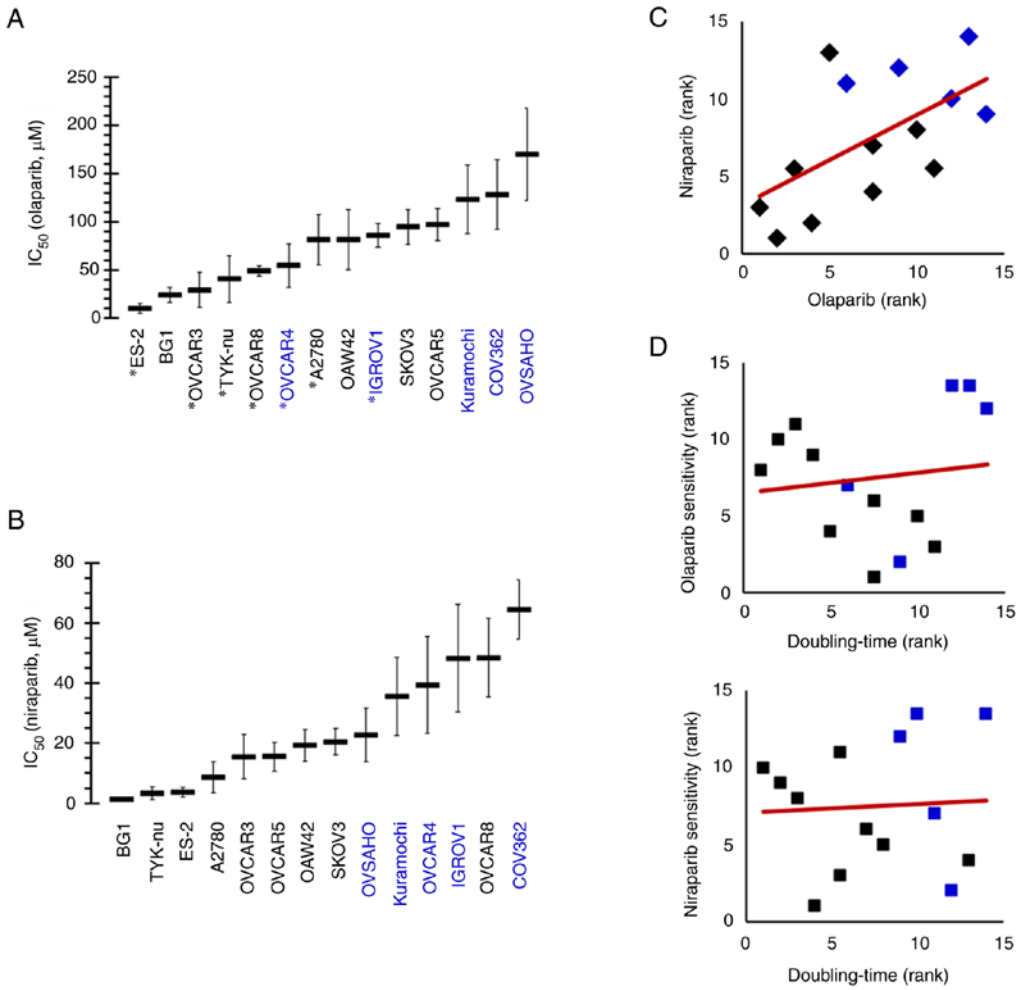

Figure 2. PARPi sensitivity (relative cell viability) and proliferation rate (doubling-time) of ovarian cancer cell line panel (n=14). (A and B) IC_{50} values (y-axis) for olaparib and niraparib of the cell lines sorted according to increasing values (x-axis). Cell lines with *BRCA*-mutations are in blue. Cell line marked with (*) in panel A were selected for the resistance acquisition protocol. (C) Spearman's rank correlation (rs) plot for olaparib sensitivity against niraparib sensitivity: rs=0.582. *BRCA*-mutated cell lines in blue. (D) Spearman's rank correlation (rs) plots for olaparib (top) and niraparib (bottom) sensitivity against the proliferation rate (doubling-time): rs=0.136 and rs=0.057, respectively. *BRCA*-mutated cell lines are presented in blue. PARPi, Poly (ADP-ribose) polymerase (PARP)-inhibitors; IC_{50}, half maximal inhibitory concentration.

spread of ovarian tumors and synergize with platinum-based chemotherapy drugs (45), although their clinical usefulness remains unclear (46). PARPi sensitivity was not only identified in cells sensitive to carboplatin and cisplatin (rs platinum compounds=0.542) but in cells sensitive to doxorubicin and epirubicin (rs anthracyclines=0.456), to paclitaxel (rs=0.667), and to romidsepsin and SAHA (rs HDACi=0.607). An association was also found between PARPi and compounds that interact with the DNA (rs=0.587) and compounds that do not interact with the DNA (rs=0.633). No correlation was found for OTS167 (rs=0.248) (Fig. 3). Details are summarized in Table II.

Next, the sensitivity to olaparib and niraparib of cells with acquired cisplatin-resistance [TYK-nu(R)

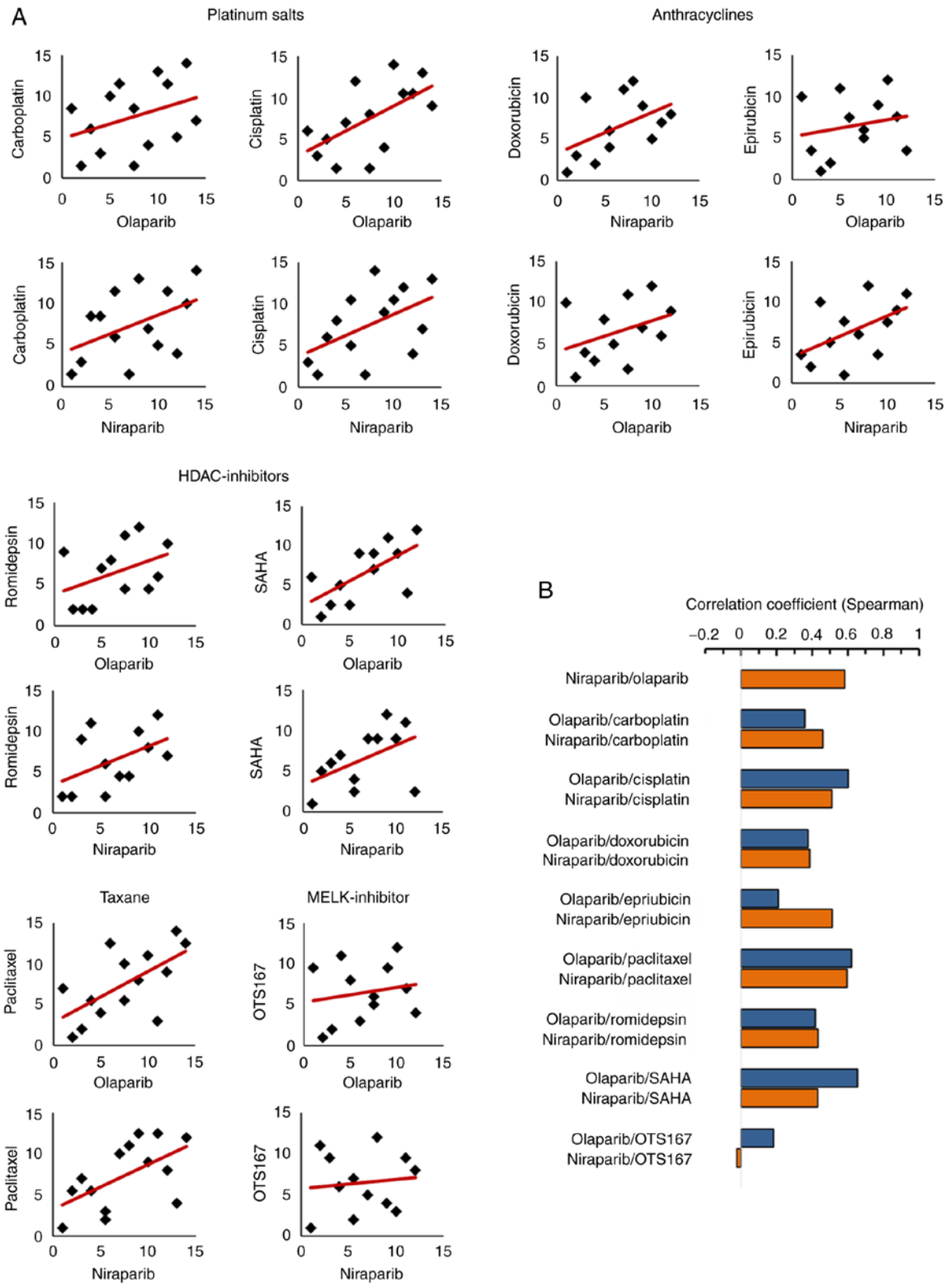

Figure 3. (A) Spearman's rank correlation plots and (B) correlation coefficients (rs) for sensitivity of olaparib or niraparib against sensitivity to platinum salts (carboplatin and cisplatin), anthracyclines (doxorubicin and epirubicin), paclitaxel, HDACi (SAHA and romidepsin), and MELKi (OTS167) are demonstrated (details in Table II). X- and Y-axis: numbers indicate the ranks of drug sensitivity for each cell line (data point). All data derived from MTT-assays (at least three independent experiments performed in quadruplets). HDACi, histone deacetylase inhibitors; MELKi, maternal embryonic leucine zipper kinase inhibitor.

and A2780/CP cells] or acquired paclitaxel-resistance (IGROV1-PXL cells) was determined. TYK-nu(R) are 4-fold and A2780/CP are >9-fold resistant to cisplatin, and IGROV1-PXL are 9.3-fold resistant to paclitaxel (37). The results (Fig. 4A) demonstrated that TYK-nu(R) cells were cross-resistant to both PARPi (4-fold to olaparib and 6-fold

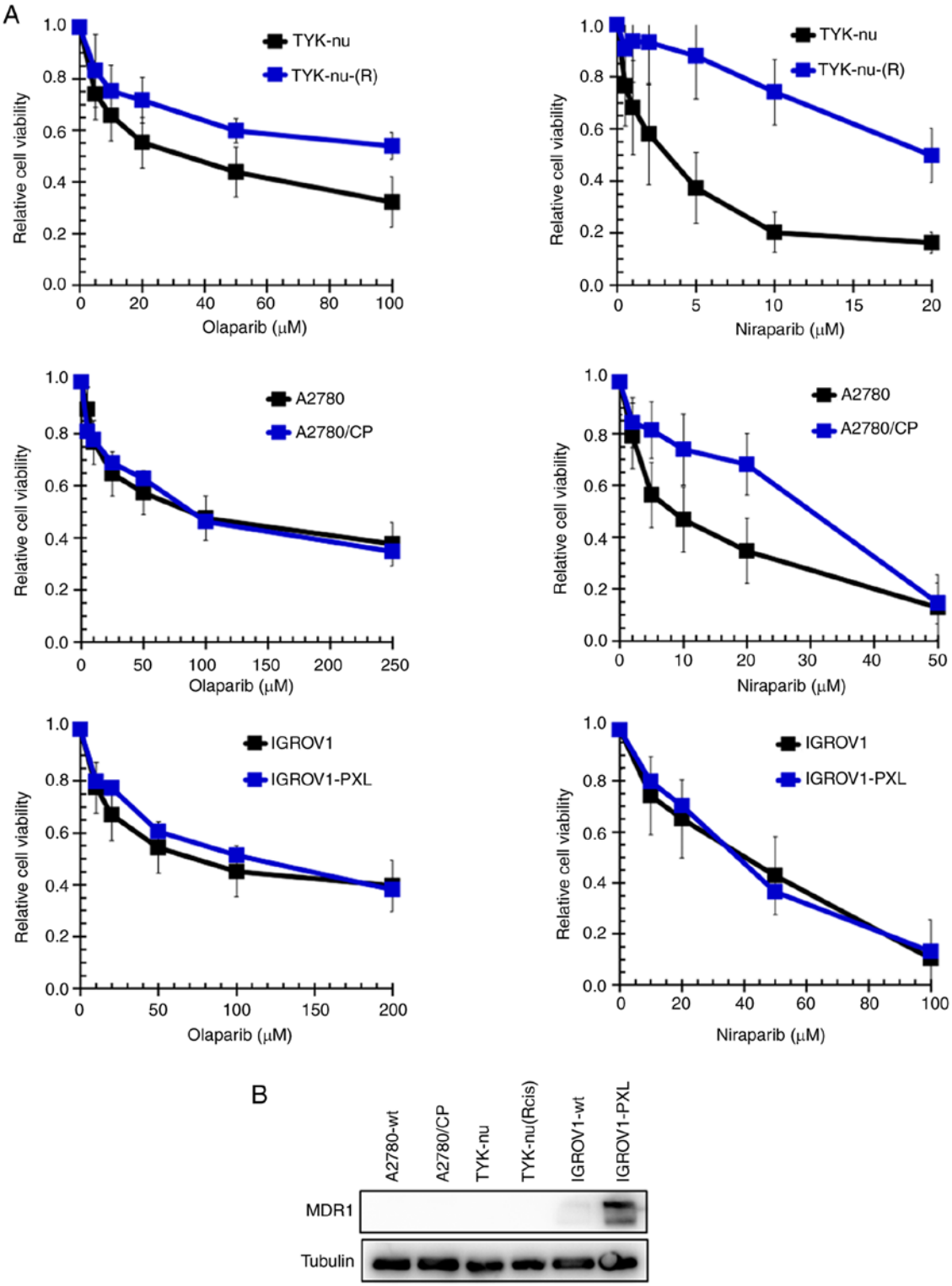

Figure 4. PARPi sensitivity of ovarian cancer cells with acquired cisplatin resistance [TYK-nu-(R), A2780/CP] or paclitaxel resistance (IGROV1-PXL). (A) Sensitivity to olaparib (left) and niraparib (right). All data are derived from MTT-assays (three independent experiments performed in quadruplets). (B) MDR1 expression in the cell lines: the lower band is the correct one for MDR1 (molecular weight ranging from 140-180 kDa, depending on its glycosylation status). PARPi, Poly (ADP-ribose) polymerase (PARP)-inhibitors; MDR, multidrug resistance.

to niraparib), while A2780/CP cells were cross-resistant to niraparib only (3.1-fold). By contrast, IGROV1-PXL cells retained sensitivity to both olaparib and niraparib. IGROV1-PXL cells express MDR1, but cisplatin-resistant A2780/CP and TYK-nu(R) cells do not (Fig. 4B). Details are shown in Table III.

Olaparib does not induce acquired resistance to PARPi or acquired cross-chemoresistance. Expanding on the previous studies which reported opposing results on PARPi-imposed resistance acquisition (31-33), seven cell lines differing in their status for *TP53*, *BRCA1* and *BRCA2*, and also in their intrinsic sensitivity to olaparib (from relatively sensitive to

Table II. Spearman's rank correlation between sensitivity to PARPi and other compounds.

Spearman's rank correlation (rs)	Olaparib	Niraparib	Olaparib + niraparib
Olaparib		0.582	
Niraparib	0.582		
Carboplatin	0.358	0.460	
Cisplatin	0.602	0.510	
Platinum			0.542
Doxorubicin	0.376	0.386	
Epirubicin	0.210	0.513	
Anthracyclines			0.456
Paclitaxel	0.622	0.597	
Taxanes			0.667
Romidepsin	0.418	0.434	
SAHA	0.654	0.432	
HDACi			0.607
OTS167	0.182	-0.024	
MELKi			0.248
DNA[a]	0.458	0.589	0.587
non-DNA[b]	0.631	0.611	0.633
ALL[c]	0.490	0.610	0.656

[a]DNA-interacting compounds: carboplatin, cisplatin, doxorubicin and epirubicin. [b]DNA-noninteracting compounds: paclitaxel, romidepsin, SAHA and OTS167. [c]All PARPi-unrelated compounds.

Table III. Olaparib/niraparib sensitivity of cells with acquired cisplatin/paclitaxel resistance.

Cell line	Olaparib (μM)	Niraparib (μM)
TYK-nu	40.6±24.9	3.3±2.0
TYK-nu-(R)	163.3±40.4	19.5±4.5
Ratio	4.0	6.0
P-value	0.019	0.016
A2780	81.3±31.0	8.7±5.1
A2780/CP	85.1±13.3	29.7±5.6
Ratio	1.05	3.1
P-value	0.814	0.001
IGROV1	80.4±26.1	43.0±17.8
IGROV1-PXL	98.8±38.5	41.8±28.6
Ratio	1.23	0.97
P-value	0.433	0.940

IC_{50}-values are presented as the mean ± SD, determined from MTT-assays. P-values calculated by the two-sided Student's t-test. Ratio, resistance factor calculated from the IC_{50}-values of the resistant vs. the parental cells.

Table IV. Proliferation rates (doubling-times).

Cell line	Time (h)	Ratio (OLA/par)	P-value
ES-2	35.4±7.9		
ES-2-OLA	30.4±4.8	0.86	0.003
IGROV1	23.7±3.6		
IGROV1-OLA	27.7±8.7	1.17	0.015
OVCAR3	43.6±9.8		
OVCAR3-OLA	48.1±8.3	1.10	0.480
OVCAR4	32.9±10.4		
OVCAR4-OLA	27.3±5.1	0.83	0.020
OVCAR8	25.3±2.5		
OVCAR8-OLA	27.6±3.4	1.09	0.139
A2780	21.5±2.6		
A2780-OLA	23.8±2.3	1.11	0.060
TYK-nu	36.6±7.4		
TYK-nu-OLA	34.8±3.7	0.95	0.747

Doubling-time presented as the mean ± SD. Ratio calculated from the doubling-times of the subcells exposed to escalating olaparib (-OLA) vs. their parental cells. P-values calculated by the two-sided Student's t-test.

relatively resistant) were selected (Figs. 1 and 2A). These cell lines were subjected to the protocol of 'repeated exposure with olaparib concentration escalation' as described in 'Materials and methods', yielding the following subcell lines: A2780-OLA, ES-2-OLA, IGROV1-OLA, OVCAR3-OLA, OVCAR4-OLA, OVCAR8-OLA and TYK-nu-OLA. The proliferation rate and the sensitivity to olaparib, niraparib, carboplatin, cisplatin, doxorubicin and paclitaxel were also determined, demonstrating that the parental cells and their matched subcells have comparable proliferation rates (Table IV).

The MTT-assay results demonstrated that in none of these seven cell lines, stepwise 48 h-exposure of cells to escalating olaparib produced acquired PARPi resistance or an acquired cross-resistance to platinum compounds, doxorubicin, and paclitaxel (Fig. 5A), meaning that resistance factors (ratio from the IC_{50}-values of the resistant subcells vs. the parental cells) >2.0 were not found. Detailed data are summarized in Table V. The failure to produce acquired PARPi-resistance was confirmed by CFAs for A2780, IGROV1, OVCAR3 and OVCAR8 (cell lines that form distinct colonies rather than proliferating as a confluent monolayer), where the clonogenic potential of the-OLA cells was not different from that of their parental cells in response to olaparib (Fig. 5B). The absence of an acquired resistance phenotype in all cells in this setup also indicated that it was not relevant whether or not *TP53* and/or *BRCA* were mutated. OVCAR8 cells were also continuously (instead of 48 h as for olaparib) exposed to three cycles of escalating Niraparib and resistance acquisition to Niraparib, Olaparib, Carboplatin and Doxorubicin was not observed (data not shown).

Genomic deletion of PARP1 does not affect sensitivity to PARPi and other chemotherapeutic compounds. To determine whether the abundance of PARP1 protein expression

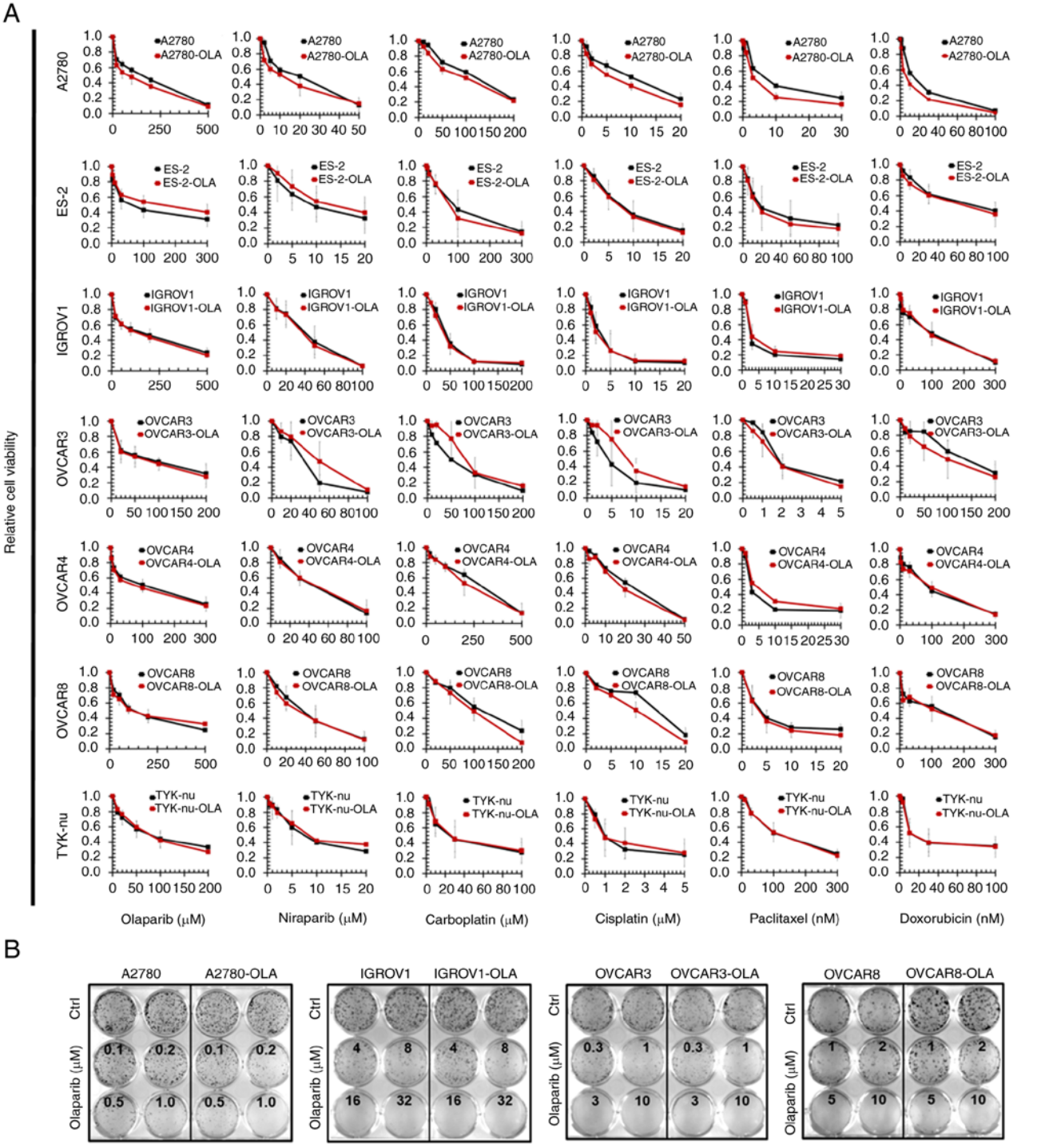

Figure 5. (A) Sensitivity to olaparib and niraparib and cross-sensitivity to carboplatin, cisplatin, paclitaxel and doxorubicin of subcells exposed to escalating olaparib (red) vs. their parental cells (black). Y-axis, relative cell viability; X-axis, drug concentration. Data (mean ± SD) are derived from MTT-assays of at least three independent experiments performed in quadruplets. (B) Representative images from colony formation assays for A2780, IGROV1, OVCAR3 and OVAR8 parental cells (left side of plate) and their respective-OLA cells (right side of plate). Top row shows untreated control (Ctrl) cultures. Numbers indicate the concentration of olaparib (μM).

affects drug sensitivity, *PARP1*-knockouts of three ovarian cancer cell lines with differential olaparib-sensitivity (OCVAR3 > OVCAR8 > OVCAR5) were produced using the CRISPR-*Cas9* technology. To this aim, three different sgRNAs targeting exons 1 and 2 were designed in order to perform CRISPR-*Cas9*-mediated mutagenesis of *PARP1* in these three cell lines (Fig. 6A). Downstream western blot analysis demonstrated reduction of PARP1 protein expression in all three *PARP1*-knockout cell lines (*PARP1*) in comparison with the non-transduced (control) and the *AAVS1*-transduced (mock) cells (Fig. 6B). The gene-editing of the *PARP1* loci was further confirmed by the TIDE assay (42) (Fig. 6C).

Sensitivity of the OVCAR3, OVCAR5 and OVCAR8 *PARP1*-knockout cells and their respective mock cells to olaparib and niraparib, carboplatin and cisplatin, doxorubicin and paclitaxel was determined by MTT-assays. The results

Table V. Drug sensitivity (IC_{50}-values) of subcells exposed to escalating olaparib.

	Olaparib (μM)	Niraparib (μM)	Carboplatin (μM)	Cisplatin (μM)	Paclitaxel (nM)	Doxorubicin (nM)
A2780	150.8±29.1	20.4±3.3	130.8±18.5	10.8±1.0	6.1±3.9	15.1±6.8
A2780-OLA	86.3±47.2	14.6±7.6	103.5±8.5	6.9±1.1	3.3±1.5	6.9±1.7
Ratio	0.57	0.72	0.79	0.64	0.54	0.46
P-value	0.068	0.236	0.052	0.002	0.255	0.092
ES-2	71.4±56.6	13.5±12.0	90.6±49.5	6.6±1.2	31.6±33.5	102.6±70.8
ES-2-OLA	185.7±75.9	16.0±8.6	73.4±27.3	6.2±2.6	20.0±14.7	99.8±69.5
Ratio	2.60	1.18	0.81	0.94	0.63	0.97
P-value	0.00003	0.493	0.442	0.752	0.895	0.307
IGROV1	166.5±68.2	39.0±15.5	72.7±47.5	4.88±2.62	2.4±0.1	99.3±49.5
IGROV1-OLA	135.6±51.0	38.0±8.3	83.3±28.4	8.17±2.50	3.3±1.6	90.0±21.4
Ratio	0.81	0.98	1.15	1.67	1.38	0.91
P-value	0.952	0.846	0.528	0.01	0.546	0.748
OVCAR3	84.0±24.0	34.9±3.6	41.3±10.6	2.42±0.77	1.8±0.3	120.0±31.7
OVCAR3-OLA	70.6±54.9	51.9±14.1	36.6±7.3	2.34±0.82	1.6±0.4	110.0±75.7
Ratio	0.84	1.48	0.89	0.97	0.89	0.92
P-value	0.261	0.0004	0.450	0.877	0.653	0.771
OVCAR4	114.0±46.2	32.4±14.0	287.5±53.0	22.3±3.2	9.1±11.1	89.3±16.8
OVCAR4-OLA	82.6±33.1	33.2±11.9	235.0±91.9	18.3±3.2	9.5±9.2	102.7±36.7
Ratio	0.72	1.02	0.82	0.82	1.04	1.15
P-value	0.255	0.925	0.572	0.336	0.97	0.61
OVCAR8	124.0±35.3	39.8±18.7	115.3±25.9	13.4±1.1	3.5±1.7	150.7±9.3
OVCAR8-OLA	140.0±51.0	34.8±13.4	93.3±25.2	10.9±3.6	3.4±1.5	115.3±66.0
Ratio	1.13	0.87	0.81	0.81	0.99	0.88
P-value	0.547	0.607	0.351	0.347	0.981	0.728
TYK-nu	87.3±40.5	8.0±3.7	33.8±25.7	1.19±0.52	17.1±11.2	110.5±41.7
TYK-nu-OLA	96.3±55.3	9.5±5.8	43.0±42.4	1.89±1.64	26.9±25.6	127.5±60.1
Ratio	1.10	1.18	1.27	1.59	1.58	1.15
P-value	0.883	0.734	0.822	0.652	0.686	0.777

IC_{50}-values presented as the mean ± SD, determined from MTT-assays. Ratio, resistance factor calculated from the IC_{50}-values of the subcells exposed to escalating Olaparib (-OLA) vs the parental cells. P-value calculated using the Student's t-test. $IC_{50,}$ half maximal inhibitory concentration.

revealed that loss of PARP1 in these cell lines does not affect sensitivity to these drugs (Table VI).

Discussion

In the present study, the potential of olaparib to induce acquired resistance to PARPi and to PARPi-unrelated compounds was investigated. The results demonstrated i) that olaparib exposure did neither induce acquired resistance to PARPi nor induce cross-resistance to PARPi-unrelated compounds such as platinum salts, paclitaxel and doxorubicin; ii) that intrinsic PARPi resistance not only associates with resistance to platinum salts but also with resistance to other chemotherapeutic compounds like doxorubicin and epirubicin, paclitaxel and romidepsin and SAHA; and that iii) cells with acquired cisplatin-resistance are PARPi cross-resistant.

The key finding of the present study is the failure to generate subcells with a detectable acquired resistance phenotype both to PARPi and to PARPi-unrelated compounds after escalating olaparib (Table V). This is consistent with a previous study reporting that long-term treatment with niraparib did not cause genetic alterations and did not increase the mutation load in wildtype and *BRCA1*-defective breast cancer cells to allow the genetic evolution of resistance (33).

The present findings are, however, opposed to two other studies reporting the occurrence of acquired PARPi resistance after long-term exposure of ovarian cancer cells to olaparib (31) and in high-grade serous ovarian carcinoma, patient-derived xenograft modes following treatment with niraparib (32). In the former study, olaparib-imposed acquired resistance to olaparib and niraparib was associated with the activation of the Wnt-signaling pathway. This was found in PEO1 cells, which are mutated in both *TP53* and *BRCA2* (and hence were cells

Table VI. Drug sensitivity (IC_{50}-values) of parental and PARP1-knockout cells.

	Olaparib (μM)	Niraparib (μM)	Carboplatin (μM)	Cisplatin (μM)	Paclitaxel (nM)	Doxorubicin (nM)
OVCAR3-mock	110.0±85.44	29.7±7.6	27.4±12.9	2.4±2.2	3.5±1.4	32.3±25.8
OVCAR3-ko (*PARP1*)	112.7±96.0	36.0±8.2	25.6±11.4	2.4±2.2	3.5±0.4	20.0±433.0
Ratio	1.02	1.18	0.94	0.99	1.01	0.63
P-value	0.973	0.493	0.845	0.986	0.972	0.518
OVCAR5-mock	307.3±169.1	34.0±3.0	154.0±18.3	6.6±1.2	2.4±0.1	99.3±49.5
OVCAR5-ko (*PARP1*)	276.7±140.1	36.3±6.0	111.7±20.6	6.5±2.1	3.3±1.6	90.0±21.4
Ratio	0.90	1.07	0.73	0.98	1.38	0.91
P-value	0.821	0.592	0.057	0.947	0.546	0.748
OVCAR8-mock	495.0±261.6	51.5±17.0	252.5±103.7	13.2±4.3	16.7±15.8	1060±1166
OVCAR8-ko (*PARP1*)	392.5±83.4	45.0±10.2	198.0±58.8	12.9±2.2	10.6±8.2	513±344
Ratio	0.79	0.87	0.78	0.98	0.63	0.48
P-value	0.501	0.544	0.405	0.922	0.591	0.507

IC_{50}-values presented as the mean ± SD, determined from MTT-assays. Ratio, resistance factor calculated from the IC_{50}-values of the *PARP1*-knockout [ko(*PARP1*)] cells vs. the *AAVS1* (mock) cells. P-value calculated by the Student's t-test. IC_{50}, half maximal inhibitory concentration.

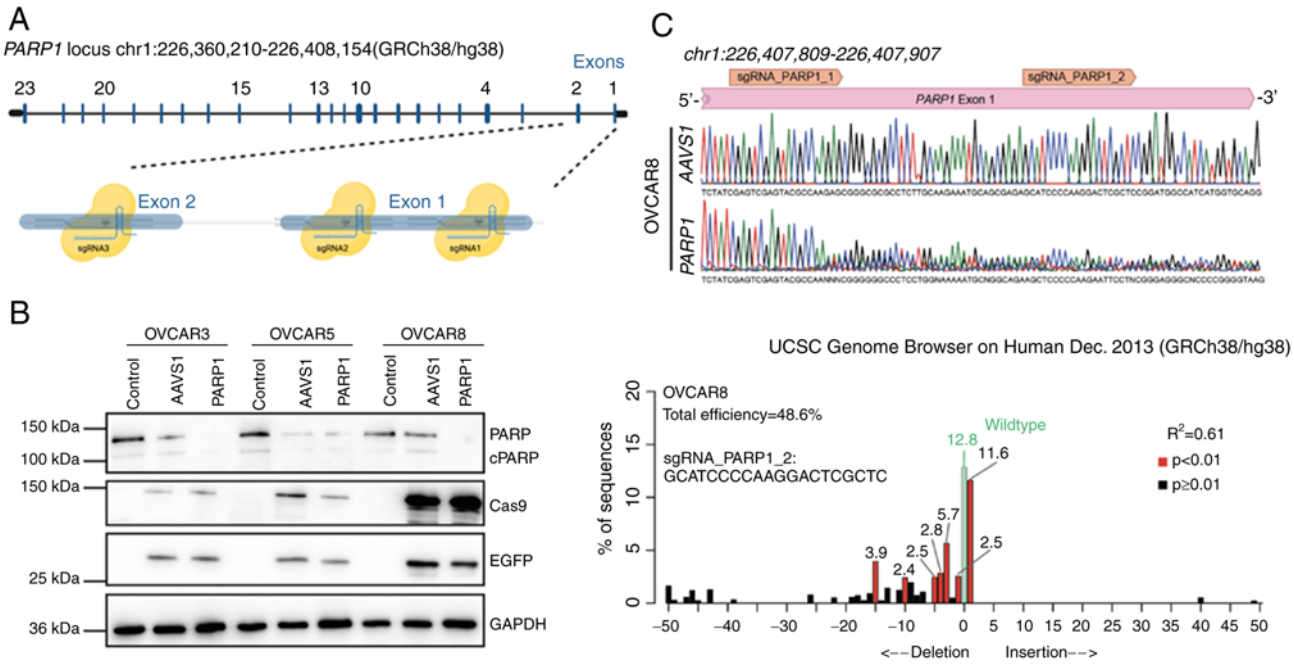

Figure 6. Establishment of CRISPR-*Cas9* meditated *PARP1* knockout in ovarian cancer cells. (A) Depiction of the CRISPR-*Cas9*-mediated mutagenesis for the *PARP1* gene (black line) using three different sgRNAs targeting the exons 1 and 2 of *PARP1*. (B) Western blotting showing the reduction of PARP1 protein expression in EGFP-enriched and Cas9-expressing OVCAR3, OVCAR5 and OVCAR8 cells that harbor the sgRNAs targeting *PARP1*. 'Control' are non-transduced cells, '*AAVS1*' are *AAVS1*-transduced (mock) cells, and 'PARP1' are PARP1-transduced cells. GAPDH is the sample loading control. (C) Representative example of *Cas9* activity in OVCAR8 cells accessed by sanger DNA sequencing, showing the percentage of indels in the targeted loci using the TIDE analysis (ref. 42). PARP, Poly (ADP-ribose) polymerase (PARP); sgRNA, single guide RNA; TIDE, Tracking of Indels by Decomposition.

representative for ovarian cancer) as well as in OVCA433 cells, which were *TP53*-mutated but *BRCA2*-wildt-ype. In the latter study, acquired PARPi resistance was associated with RAD51C promoter methylation loss, indicating that PARPi treatment can cause demethylation of RAD51C and that a single alteration was sufficient to confer PARPi resistance (32). Whether olaparib-induced acquired PARPi resistance also associated with cross-resistance to PARPi-unrelated compounds was not reported in these studies (31,32).

The study by Yamamoto *et al* (31), also suggested that PARPi resistance acquisition can occur regardless of the *BRCA2*-status. It was also considered that if the status of *BRCA1*, *BRCA2*, and *TP53* in our subcell panel is relevant for resistance acquisition, but failed to produce acquired resistance in all cell lines investigated,

that is regardless of whether they are mutated in *TP53* (ES-2, OVCAR3, OVCAR8, TYK-nu) or *TP53* and *BRCA2* (IGROV1, OVCAR4) or whether they are wild-type for both (A2780). Intriguingly, not even the triple *TP53*/*BRCA1*/*BRCA2*-mutant IGROV1 cells, which carry also mutations in numerous DNA repair-associated genes including *ARID1A, ATR, BLM, MRE11, MLH1, MSH2, MSH3, MSH6, RAD50 and RAD52*, developed acquired resistance after olaparib escalation. It thus appears that whether or not olaparib-imposed resistance acquisition occurs does not necessarily depend on the *TP53* and *BRCA* mutation status and that, if it occurs, it may rather be a matter of cell line dependence and/or of the mutational background of potential oncogenic genes.

There are other important findings. One is that intrinsically PARPi-sensitive ovarian cancer cells were not only cross-sensitive to platinum salts as expected (7,47,48) but notably tended to be also cross-sensitive to anthracyclines and even to DNA-unrelated compounds like HDACi, and paclitaxel (Table II). These findings suggested an association between PARPi sensitivity and a general chemo-sensitivity of cancer cells, that is beyond platinum sensitivity.

Anthracyclines intercalate into DNA and directly interfere with topoisomerase II, resulting in DSB and eventually in cell death (49). *BRCA*-deficient cells have been revealed to be sensitive to doxorubicin (50) and doxorubicin may reduce PARP activity and PARP1 expression (51). It appears that doxorubicin can mimic the effect of PARPi by reducing the abundance and function of PARP. Similarly, HDACi may acetylate PARP and increase PARP binding to chromatin, resembling PARP-trapping to DNA and hence mimicking the effect of PARPi, which eventually leads to decreased repair of cytotoxic DSBs (52) or HDACi induce hyperacetylation of the nuclear HSP90 and cause depletion of HR-related proteins, thus conferring *BRCA*ness and defective DNA damage and HR response in wild-type *BRCA1* breast cancer cells (53). Paclitaxel interacts with microtubules and inhibits cytokinesis and is not known to be implicated in DNA-repair (54). PARP has also various functions in mitosis, and PARP inhibition may give rise to various mitotic defects (55), possibly imitating the effect of the microtubule poison.

Similarly, cells with acquired platinum-resistance were cross-resistant to PARPi, whereas cells with acquired paclitaxel resistance remain PARPi-sensitive (Table III). The absence of PARPi cross-resistance in cells with acquired paclitaxel-resistance is obvious as PARPi and paclitaxel act by different mechanisms. Moreover, the observed PARPi cross-resistance in cells with acquired cisplatin-resistance is predictable since resistance to platinum-based chemotherapy is a strong predictor for PARPi resistance (12). However, it is unclear why A2780/CP cells were cross-resistant to niraparib but not olaparib, whereas TYK-nu(R) cells are cross-resistant to both. Although olaparib was reported to be a MDR1 substrate (56), an involvement of MDR1 does not explain the present observations. Cisplatin-resistant A2780/CP and TYK-nu(R) cells and their respective parental cells do not express MDR1, indicating that PARPi cross-resistance in A2780/CP and TYK-nu(R) cells is MDR1-independent. Similarly, MDR1-expressing paclitaxel-resistant IGROV1-PXL cells retain PARPi sensitivity, also indicating that MDR1 is not involved.

Another interesting observation was that at least in our cell line panel cells with *BRCA*-mutations tended to be PARPi-resistant rather than PARPi-sensitive as compared with cell lines with no *BRCA* mutations, which is opposed to the synthetic lethality concept (3,4).

Taken together, our *in vitro* results argue against olaparib as a likely inducer of acquired PARPi resistance and cross-resistance to other chemotherapeutic compounds. Not ignoring that the *in vivo* and *in vitro* situation may be different, but nevertheless assuming certain transferability into a clinical context, the present results would mean that an olaparib-based therapy would not produce PARPi- or chemotherapy-resistant cells and that 'any other' chemotherapy or even a therapy with a different type of PARPi could follow a PARPi-based therapy. They also suggested to extend the current view of PARPi efficacy into a broader context, that is beyond *BRCA*ness, meaning that PARPi can be an option to treat cancers regardless of a *BRCA*ness phenotype. However, this is rather speculative and should be evaluated in clinical trials. Moreover, although not observed, an acquired hypersensitivity phenotype would be even more intriguing in this respect and perhaps be an add-on to the idea that so-called 'exceptional responders' may be an alternative strategy to better identify novel molecular determinants of (hyper)sensitivity to these agents PARPi (57).

The failure to induce acquired PARPi resistance seems predictable, because PARPi are, in contrast to platinum compounds (58), unlikely mutagenic. On the other hand, it cannot be ruled out that other genetic changes or cellular events may occur (33) which in our experimental setup remain phenotypically undetectable, maybe because the present experimental protocol was not sufficiently stringent to produce or to select for subcells with acquired resistance. Similarly, it appears too simple to just consider *BRCA*- and *TP53*-mutations or the HRD-status (10) to delineate the sensitivity of different drugs to PARPi and to other chemotherapeutics. Rather, the entire mutational profile of the potential oncogenes for each individual cell line should be taken into consideration.

Acknowledgements

Not applicable.

Funding

The present study was in part supported by the Swiss National Science Foundation (grant no. CRSII5_171037).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AF mainly conceived the study, performed the experiments, wrote and edited the manuscript. NM, AT and MD performed the experiments. RC performed the experiments and edited the manuscript. FJ and VHS made substantial contributions to the conception and design of the study and critically reviewed and edited the manuscript. FJ and RC confirm the authenticity of

all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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