

Synthetic Lethality Triggered by Combining Olaparib with BRCA2–Rad51 Disruptors

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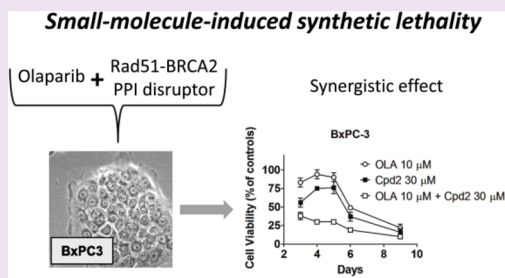
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Supporting Information

ABSTRACT: In BRCA2-defective cells, poly(adenosine diphosphate [ADP]-ribose) polymerase inhibitors can trigger synthetic lethality, as two independent DNA-repairing mechanisms are simultaneously impaired. Here, we have pharmacologically induced synthetic lethality, which was triggered by combining two different small organic molecules. When administered with a BRCA2–Rad51 disruptor in nonmutant cells, Olaparib showed anticancer activity comparable to that shown when administered alone in BRCA2-defective cells. This strategy could represent an innovative approach to anticancer drug discovery and could be extended to other synthetic lethality pathways.



Synthetic lethality is the lethal phenotype arising from a combination of two gene mutations, which do not affect cell viability when they occur individually.^{1,2} In principle, targeting the synthetic lethal partner of an altered gene in cancer should selectively kill cancer cells while sparing normal cells. One straightforward application of synthetic lethality in anticancer drug development is the use of poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) inhibitors in BRCA2-defective oncology patients. BRCA2 mutations are undesirable because they are associated with tumorigenesis (the so-called “BRCAness” tumors).³ However, they can impair DNA-repairing mechanisms and improve cancer cells’ sensitivity to DNA-damaging chemotherapeutics and PARP inhibitors.⁴ Olaparib (Lynparza, developed by AstraZeneca) was the first PARP inhibitor approved in 2014 to treat women with advanced ovarian cancer associated with defective BRCA genes.⁵ Several clinical trials are studying Olaparib for breast and pancreatic BRCAness tumors, and these strategies rely on the synthetic lethality paradigm.^{6,7} Inhibiting PARP enzymes allows the accumulation of single-strand breaks, which are subsequently converted into DNA double-strand breaks (DSBs). Normal cells can efficiently repair DSBs. But when homologous recombination (HR) is impaired, as with BRCA-ness tumors, DSBs cannot be mended, leading to the selective

killing of cancer cells.⁸ Of the core proteins involved in HR, Rad51 is an evolutionarily conserved recombinase enzyme that plays a key role in recombination. It multimerizes on the 3′-tailed single-strand DNA overhangs, forming nucleofilaments that are responsible for seeking homologous DNA sequences and subsequent strand-exchange invasion.⁹ Rad51 is recruited to the DSB site by BRCA2 through a protein–protein interaction mediated by eight well-conserved motifs, known as BRC repeats.^{10–12} In BRCA2-defective cells, impaired recruitment of Rad51 means that HR is not properly performed, and so DSBs are not efficiently repaired.

Here, we report on a new anticancer drug discovery concept, which combines BRCA2–Rad51 disruptors with Olaparib in order to trigger synthetic lethality exclusively using small organic molecules. To identify disruptors of this protein–protein interaction, we performed a virtual screening protocol based on high-throughput docking using the X-ray crystal structure of BRC4 (the fourth BRC repeat) in complex with the catalytic domain of Rad51 (PDB code: 1N0W).¹³ BRC4 binds Rad51 in two different hydrophobic pockets (Figure 1a).^{14,15}

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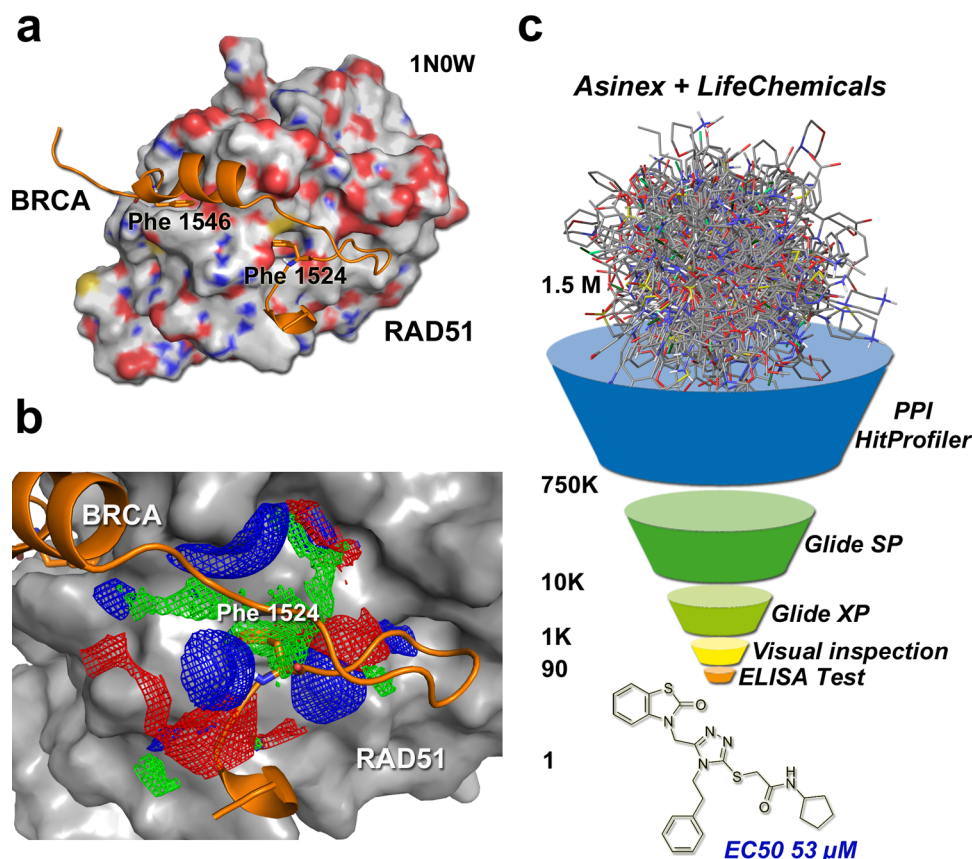


Figure 1. (a) Rad51–BRCA2 BRC repeat complex. Rad51 is represented as a surface, BRCA as a cartoon. The two hot spots of the interaction between the proteins (Phe1524 and Phe1546) are highlighted in sticks. (b) SiteMap hydrophobic (green), hydrogen-bond donor (blue), and hydrogen-bond acceptor (red) maps for 1N0W in the proximity of Phe1524. (c) Schematic representation of the virtual screening protocol. The numbers on the left column represent the number of compounds in each step; the filters used to narrow down the compounds are reported on the right column.

One pocket can lodge BRC4's "FxxA" motif and is critical for Rad51 multimerization (Phe1524 of BRC4 or Phe86 of Rad51 can both bind into this pocket). The other pocket can lodge the "LFDE" motif far from the oligomerization interface. High-throughput docking was carried out using the "FxxA" pocket (Figure 1b), according to the protocol reported in Figure 1c. Ninety small molecules were selected, purchased, and tested for their inhibitory activity using a competitive biochemical ELISA assay, as previously described by Rajendra *et al.*¹⁴ This assay is an efficient tool for directly measuring inhibition of the BRC4–Rad51 interaction, at a molecular level. Indeed, when one of our compounds physically interacts with Rad51, BRC4 is displaced as a consequence of the disruption of the BRC4–Rad51 molecular interaction.

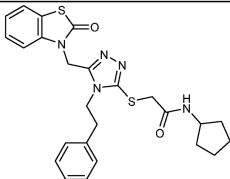
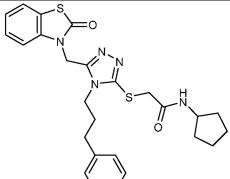
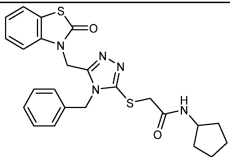
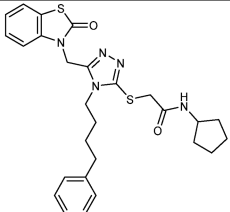
Of the tested compounds, **1** (Figure 1c and Table 1) was the best candidate in terms of EC₅₀ and chemical tractability, and its activity was confirmed by retesting the newly synthesized compound **1**. Preliminary structure–activity relationship (SAR) studies focused on the ethyl phenyl region to find the proper length of the alkyl chain between the triazole and the phenyl ring. The synthesis of compounds **1–4** is reported in the Supporting Information. A large library of analogues along with extensive SAR studies will be described in a subsequent medicinal chemistry paper. Compound **2** (Table 1), with a propyl phenyl fragment, showed slightly better biological activity than the initial hit compound and was selected for further biological studies.

To test the hypothesis of pharmacologically induced synthetic lethality, we studied whether the combination of Olaparib with **2** could increase the efficacy of PARP inhibitors in cells expressing the functional BRCA2 protein. These results were compared to those obtained using BRCA2-defective cells. Given pancreatic cancer's lethality and the availability of a cell line lacking a functional BRCA2 (the Capan-1 cell line), we selected pancreas adenocarcinoma as a cellular model. The functional BRCA2 cell line was BxPC-3 because, aside from the BRCA2 mutation, these cells match Capan-1 well in terms of tumor type and differentiation state.¹⁶ First, we evaluated Olaparib's effects on the viability of the two cultures. The cell lines were exposed to scalar doses of the drug for 1–7 days. The effects observed at day 7 are summarized in Figure 2a. As expected, Olaparib was more active against BRCA2-defective cells (*i.e.*, Capan-1) relative to BxPC-3. We then selected a 10-μM concentration to investigate the drug in combination with **2**.

Figure 2b and c show the effects caused by **2** on the viability of BxPC-3 and Capan-1 cells, respectively. The compound was tested at scalar doses (0–50 μM) for a period of 7 days. Notably, the culture expressing the functional BRCA2 appeared to be more responsive to the compound.

Then, we focused on the combination of Olaparib with **2**. Figure 2d,e report the results. In Capan-1 cells, the combination with **2** did not affect Olaparib toxicity. Conversely, in BxPC-3 cells, administering **2** at concentrations of 20 μM

Table 1. EC₅₀ of 1, 2, 3, and 4 on ELISA Assay^a

Compound	Structure	EC ₅₀ [*]
1		EC ₅₀ = 53 ± 3 μM
2		EC ₅₀ = 25 ± 2 μM
3		N.A. ^{**}
4		N.A. ^{**}

^a(*) All points were tested in triplicate with error bars indicating the standard deviation. (**) Not active.

and 30 μM significantly increased the drug's effects. In these samples, evaluation of the combination index suggested a potentially synergistic effect rather than a simple additive effect between the two molecules (Figure S1). This was likely due to the simultaneous impairment of two DNA repair mechanisms, eventually resulting in a pharmacologically induced synthetic lethality. Similar results were recently obtained by Jdey *et al.*, who investigated oligonucleotides in combination with Olaparib and other PARP inhibitors.¹⁷

To strengthen the evidence that 2 inhibits the BRCA2–Rad51 signaling pathway, cell viability experiments were also performed on BxPC-3 cells with stable Rad51 silencing (Figure S2). Results were evaluated after 96 h of incubation with the compounds, when the highest effects had been observed on the parental culture (Figure 2d). As reported in Figure 2f, abrogation of Rad51 expression in BxPC-3 cells was found to cancel the potentiating effect produced by 2 on Olaparib cytotoxicity.

In further studies, we verified whether administering 2 increased the DNA damage signs produced in Capan-1 and BxPC-3 cells by Cisplatin. DNA damage was evaluated by assessing H2AX phosphorylation (γH2AX). Cell cultures were exposed to 12 μM Cisplatin for 1 h. The drug was then removed, and 2 (20 μM and 30 μM) was added to the culture medium. We preliminarily verified that 1 h treatment with 12 μM Cisplatin caused DNA damage signs, which became evident

within 24 h and tended to reduce during the following 24 h. In the cell cultures exposed to Cisplatin plus 2, DNA damage was evaluated at 48 h. Figure 2g shows the densitometric reading of phospho-H2AX levels, evidenced by immunoblotting. The vertical lines indicate the samples for comparison. The blotted membrane image can be seen in Figure S4. In Capan-1 cells, 2 did not affect Cisplatin-induced DNA damage. Conversely, an increased γH2AX signal was observed in the BxPC-3 cell samples treated with the 2/Cisplatin combination. Although not statistically significant, this increase is in line with data obtained in the cytotoxicity experiments for the 2/Olaparib combination. We also sought to demonstrate that 2's effect (concentration of 20–30 μM) on γH2AX activation could be similar to that of Rad51 gene knockdown. A procedure for transient Rad51 silencing was adopted in this case (Figure S3). Results are shown in Figure 2h. The presence of γH2AX indicates that some of the DNA damage remains unrepaired and may contribute to cancer cell death. Constitutive DNA damage without any genotoxic insult can be present to a varying extent in cancer cell lines.¹⁸ Rad51-defective cells exhibited a marked increase in the frequency of spontaneous and chromatid type breaks, indicating Rad51's vital role in the DSB repair pathway.¹⁹ Here, γH2AX focus formation increased significantly after Rad51 gene silencing ($p < 0.05$, Figure 2i). Figure 2i also shows that the effect of Rad51 gene silencing on γH2AX was comparable to that of treatment with 2. No statistically significant difference was observed in the number of γH2AX-marked nuclei between the Rad51-silenced and the 2-treated cultures. Finally, to assess potential off-target activities of 2, a few proteases and phosphatases were selected and tested, as related structures have the potential to inhibit these enzyme families. As shown in Table 2, compound 2 was rather clean and totally inactive against a set of selected proteases and phosphatases, which could play a role in the cell-based anticancer activity here investigated.

Clinical evidence has shown that PARP inhibitors are more effective in oncology patients carrying BRCA2 mutations than in nonmutated individuals.²⁰ Mutated BRCA2 protein partially loses its ability to bind Rad51, resulting in incompetent repair of damaged DNA in cancer cells.¹³ As such, we hypothesized that the enhanced sensitivity in BRCA2-defective oncology patients could be mimicked pharmacologically in nonmutated individuals by administering disruptors of the BRCA2–Rad51 interaction. This would make cancer cells more sensitive to PARP inhibitors, widening the spectrum of possible applications of these anticancer drugs. We have here demonstrated that combining Olaparib with a small molecule BRCA2–Rad51 disruptor does indeed increase the sensitivity of nonmutated pancreatic cancer cells, making them as responsive to Olaparib as BRCA2-defective cell lines. Furthermore, our analyses have shown a synergistic effect (rather than a simple additive effect) between BRCA2–Rad51 disruptors and Olaparib, which is likely a consequence of the simultaneous inhibition of two DNA-repair mechanisms. Oncology patients carrying BRCA2 mutations also show enhanced sensitivity to some DNA-damaging chemotherapy agents, including platinum derivatives, topoisomerase inhibitors, 5-fluorouracil, and alkylating drugs.²¹ Combining BRCA2–Rad51 disruptors with these drugs could improve efficacy in treating nonmutated patients, broadening the potential applications of these drugs in anticancer chemotherapy. Finally, BRCA2–Rad51 disruptors could potentially be used to treat patients who develop resistance to PARP inhibitors, which is mostly due to either restoration of

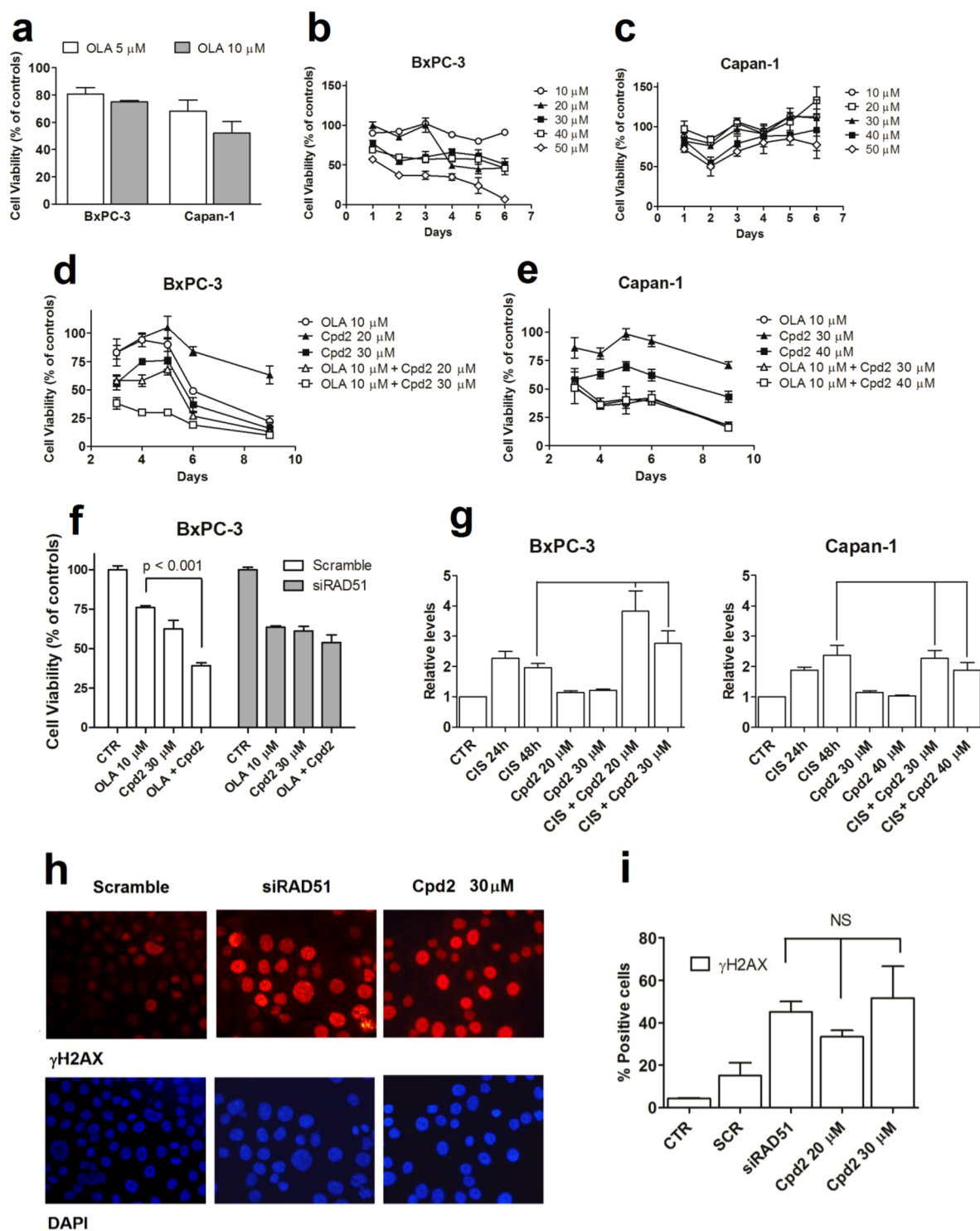


Figure 2. Experiments on cell cultures. (a) Effect of 7 days of Olaparib treatment on the viability of BxPC-3 and Capan-1 cells. (b, c) Dose–response curves assessed in BxPC-3 (b) and Capan-1 (c) cells exposed to **2**. The compound doses used for combination experiments have been sketched with dark symbols. (d, e) Combination experiments of Olaparib with **2** in BxPC-3 (d) and Capan-1 (e). In the BxPC-3 cell line, the results obtained with Olaparib were compared with those obtained with Olaparib in combination with **2** using two-way ANOVA; for both 20 and 30 μ M **2**, p values < 0.001 were obtained at 3–6 days of treatment. (f) Combination experiment of Olaparib with **2**, in BxPC-3 cells after stable Rad51 silencing, compared with scrambled controls. The effect was evaluated after a 96 h treatment. Results obtained in siRad51 cultures were statistically analyzed by ANOVA followed by Bonferroni post test. (g) Densitometric reading of immunoblots evaluating γ H2AX expression (normalized on Actin levels) in cells treated with Cisplatin (12 μ M) and **2** at the same doses used in the Olaparib combination experiments. For a detailed explanation, see the text. Vertical lines indicate the samples for comparison. (h) Immunofluorescence γ H2AX detection in nuclei of BxPC-3 cells treated with RAD51 siRNA or **2**. The percentage of positive cells are reported in the graph of frame (i).

nonmutated BRCA2 protein²² or overexpression of Rad51,²³ which in turn increases HR. Indeed, Rad51 is overexpressed in

a wide variety of cancers, including breast cancer, and is a mechanism for increasing HR in cancer cells.²⁴ Since several

Table 2. Enzyme Inhibition Activity for Compound 2 in CEREP Assays against Some Potential Off-Targets^a

enzyme	% inhibition at 25 μ M	references
cathepsin B (h)	1.9	26
cathepsin D (h)	−6.6	27
cathepsin E	−25.8	27
cathepsin S (h)	12.3	28
MMP-2 (h)	−2.9	29
phosphatase CDC25A (h)	−5	30
phosphatase CDC25B (h)	−0.3	30
protein serine/threonine phosphatase, PP2A	5.5	31
protein tyrosine phosphatase, PTPRC (CD45)	−2.9	32, 33

^aResults showing an inhibition or stimulation higher than 50% are considered to represent significant effects of the test compound.

anticancer chemotherapy agents cause DSBs, inhibiting HR could impact cancer cell survival in a major way and may increase sensitivity to DNA-damaging agents or radiotherapies.

In conclusion, we have demonstrated that, when administered in combination, Olaparib and a BRCA2–Rad51 disruptor can lead to anticancer activity, which is very similar to that observed in BRCAness tumors treated with PARP inhibitors. This new paradigm is based on the possibility to trigger synthetic lethality by exclusively using small organic molecules. In addition to opening innovative avenues for treating cancer in BRCA2-mutated and nonmutated patient populations, this paradigm could also be extended to other gene pairs involved in diverse synthetic lethality pathways.

METHODS

ELISA Assay. Competitive ELISA screening assays using biotinylated BRC4 peptide to disrupt the BRC4–RAD51 interaction were performed by modifying the method described by Rajendra and Venkitaraman.¹⁴

BRC4-biotinylated peptide (N-term BiotinKEPTLLGFHT-ASGKKVKIAKESLDKVKNLFDKEKEQ from Life Technologies) was used to coat 384 well plates (Nunc). After washing with PBS containing 0.05% Tween-20 (PBST), and blocking with the solution BSA 1% /PBST, overnight hybridization with human Rad51 protein (NP_002866 Creative Biomart, NY) was performed. Test compounds were added in dose–response from 0.01 to 100 μ M in triplicate with constant DMSO 1%. Antibody raised against Rad51 (Millipore) and HRP-secondary antibody staining to develop the 3,3',5,5'-tetramethylbenzidine signal (Sigma) quenched with 1 M HCl was used as the assay readout. Colorimetric measure was read on a Victor5 (PerkinElmer) plate Reader. BRC4 and Rad51 were included in the assay as positive controls. Results were analyzed by using GraphPad Software.

Cell Culture. BxPC-3 and Capan-1 cells were grown in RPMI 1640 containing 10% FBS, 100 U/mL penicillin/streptomycin, and 4 mM glutamine. All media and supplements were from Sigma-Aldrich.

Crystal Violet Assay. BxPC-3 and Capan-1 cells (10^3 – 10^4 /well) were plated in 96-well plates and treated. At the end of treatments, the cell culture medium was removed, and the cells were washed by PBS and fixed in glutaraldehyde 1% in PBS for 15 min at rt and rinsed with PBS several times. Then, the samples were incubated in 0.01% Crystal Violet for 30 min and washed in PBS. To each well was added 100 μ L of cold ethanol 70% and the absorbance measured in a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The results were expressed as a percentage of viable cells on the control.

Interaction Index. The interaction index between Olaparib and 2 was assessed by applying the following formula, as reported in ref 25:

[Surviving cells treated with the combination]

/[(Surviving cells treated with Olaparib)

× (Surviving cells treated with 2)]

According to ref 25, a result ranging from 0.8 to 1.2 denotes an additive effect. Synergism is indicated by a result <0.8; antagonism by a result >1.2.

Western Blot. Cell cultures (1.5×10^6 cells) were exposed to Cisplatin (12 μ M) for 1 h. Cisplatin was then removed, and 2 was added to the culture medium at the doses used in the combination experiments with Olaparib. After 48 h of incubation, cells were lysed in 100 μ L of RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). The cell homogenates were left 30 min on ice and then centrifuged 15 min at 10 000g. A total of 50 μ g of proteins of the supernatants (measured according to Bradford) were loaded into 12% polyacrylamide gel for electrophoresis. The separated proteins were blotted on a low fluorescent PVDF membrane (GE Lifescience) using a standard apparatus for wet transfer with an electrical field of 80 mA for 4 h. The blotted membrane was blocked with 5% BSA in TBS-Tween and probed with the primary antibody. The antibodies used were rabbit anti- γ H2AX [phospho S139] (Abcam) and rabbit anti-Actin (Sigma-Aldrich). Binding was revealed by a Cy5-labeled secondary antibody (anti rabbit-IgG, GE Lifescience; anti mouse-IgG, Jackson Immuno-Research). All incubation steps were performed according to the manufacturer's instructions. Fluorescence of the blots was assayed with the Pharos FX scanner (BioRad) at a resolution of 100 μ m, using the Quantity One software (BioRad).

Rad51 Silencing in BxPC-3 Cells. Both stable and transient Rad51 silencing were obtained.

For the transient silencing procedure, the sequences of small interfering RNA (siGENOME SMART pool and siGENOME Non-Targeting siRNA Pool #1) were provided by Dharmacon Research, Lafayette, Colorado, USA. Nontargeting siRNA (SCRAMBLED) and siRNA duplexes for Rad51 (10–100 nM) were transfected into BxPC3 cells using Lipofectamine 2000 (Invitrogen) for 5 h, according to the supplier's instructions.

Stable Rad51 silencing procedure:

Transduction of BxPC3 Cells. Lentiviral sh-RNA based plasmids targeting Rad51 were obtained from Sigma-Aldrich (MISSION shRNA DNA Clone TCR). The plasmids were tested for their ability to knock down Rad51 and to cooperate with Olaparib in synthetic lethality assays. Of the plasmids that were validated using these initial assays, we selected one (pLKO.1 Rad51, TRCN0000018877) for the studies described here. The sequence of the short hairpin RNA (shRNA) targeting the human Rad51 (GenBank accession no. NM_002875) was 5'-CCGGGCTGAAGCTATGTTCCG-CCATTCTCGAATGGCGAACATAGCTTCAGCTTTTT-3'. The control lentiviral pLKO.1 contains a scrambled sequence, not targeting known genes. To generate viral particles, human embryonic kidney 293 cells (ATCC) were cotransfected with the lentiviral vector and packaging plasmids (VSVg and PAX8).

BxPC3 cells were exposed to lentivirus-containing supernatant for 16 h in the presence of Polybrene (8 μ g/mL). Pools of stable transfectants were established by selection using 1 μ g/mL Puromycin for 72 h and maintained in a medium containing 1 μ g/mL of Puromycin.

Western Blot. The extent of Rad51 knockdown was measured by Western Blot. Whole cell extracts were obtained by lysis in urea buffer (8 M urea, 25 mM Tris-HCl at pH 6.8, 1 mM EDTA). Proteins were quantified (Biorad Bradford assay), and 40 μ g of total proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane (Whatman, Schleicher, and Schuell), and probed with antibodies anti-Rad51 (sc-8349) and antivinculin (Sigma-Aldrich).

siControl (Scrambled) and siRad51 BxPC-3 cells were maintained in a culture medium with 1 μ g/mL of Puromycin added.

RT-PCR. RNA was extracted by PureZOL RNA Isolation Reagent (BioRad, CA, USA). Amplification of specific PCR products was performed using the Verso one-step RT-PCR ReddyMix kit (Thermo

Fisher Scientific, Waltham, MA, USA). β -actin was used as a housekeeping gene, and the reaction was run with both Rad51 and β -actin primers into the same tube. The annealing temperature was set at 58 °C. The primer sequences were Rad51 Forward 5'-CTTTGGCCCAACCCATTTTC-3' and Rad51 Reverse 5'-ATGGCCTTTCCTTCACCTCCAC-3' and β -actin Forward 5'-ATCGTGCCTGACATTAAGGAGAAG-3' and β -actin Reverse 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. PCR products were loaded onto a 1% agarose gel, run into an electrophoresis chamber, stained by ethidium bromide, and visualized with a UV-transilluminator. Bands were analyzed with a Kodak Electrophoresis Detection and Analysis System (EDAS 290, Eastman Kodak Company, Rochester, NY, USA).

Immunofluorescence. Cells were grown on coverslips. Control, treated, and transfected cells were fixed in formalin 1% in PBS for 20 min and then in ethanol 70%, air-dried, and washed twice in PBS. The samples were incubated in 10% Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA) in PBS for 30 min at 37 °C and subsequently in the primary antibody, rabbit polyclonal antibody anti γ H2AX (1:1000 in 1% BSA in PBS), or anti Rad51 (1:500 in 1% BSA in PBS), overnight at 4 °C. After washing, the samples were incubated in antirabbit-rhodamine (1:500, 1% BSA in PBS) or antirabbit-FITC conjugated secondary antibody (1:1000 1% BSA in PBS) for 1 h at 37 °C, washed, air-dried, and mounted in a solution 1:500 DAPI in DABCO. The samples were analyzed with a Nikon fluorescent microscope equipped with filters for FITC, TRITC, and DAPI.

Enzymatic Assays. Assays on proteases and phosphatases were run by CEREP (Le Bois l'Eveque, FR), as previously described in references reported in Table 2.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00707.

Supporting Figures S1–S4, virtual screening protocol, and experimental details of all synthetic procedures together with characterization data for all intermediates and final compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This paper is dedicated to the memory of Paola (1979–2015).

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